

# **X-LINKED MENTAL RETARDATION IN S.E. SCOTLAND**

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## **DECLARATION**

I declare that:-

- a) This thesis has been composed by myself.
- b) The work is either my own or the work/author involved is clearly stated.



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If only I had a pound for every time someone said "When will it be finished?".....

## ABSTRACT

Approximately 0.3-0.4% of the general population has moderate or severe mental retardation (MR) and X-linked mental retardation (XLMR) comprises ~20-50% of all forms of MR. The existence of XLMR has been recognised since 1929 and surveys since then have confirmed the male excess of MR. In 1943, Martin and Bell reported a family with MR and an unusual pattern of inheritance; the condition they described is now known as fragile X syndrome and accounts for ~40% of all XLMR. More than 99% of fragile X syndrome is caused by expansion of a CGG repeat in the 5' UTR of the *FMRI* gene, and measurement of CGG repeat length is a diagnostic test for the syndrome. Since the cloning of the *FMRI* gene in 1991, many methods for PCR amplifying across the repeat have been published. I evaluated some of these in this laboratory and made several minor adjustments which resulted in some improvements in terms of yield of product and reduction in lane background. I concluded that the most reliable method for amplifying CGG repeats in the normal and low premutation size ranges is PCR using a fluorescent primer.

In order to determine the proportion of MR due to fragile X, CGG repeat analysis was carried out by both Southern blotting and PCR on DNA from 723 mentally retarded patients in S.E. Scotland. This resulted in the identification of 8 new cases of fragile X syndrome (~1% of the total number of referrals for developmental delay). This developmentally delayed cohort was also compared to normal and high IQ cohorts to address the possibility of a relationship between CGG repeat number and IQ variation in the general population. Three interesting prenatal diagnostic cases were studied in detail to answer important practical questions concerning the nature and timing of repeat expansion.

Well over 100 other XLMR conditions have been described, of which approximately 50% have been regionally mapped. Some of these are non-specific forms of XLMR in which MR is the only manifestation; others are XLMR syndromes with a characteristic and well defined phenotype. A family with non-specific XLMR is

described in which the proband presented as a non dysmorphic male with unexplained MR, for exclusion of fragile X syndrome. He had a CGG allele in the normal range and the MR segregating in the family was shown not to be linked to *FMR1*. Subsequent analysis of polymorphic X-linked markers showed linkage to Xq21.

A new XLMR syndrome is represented by a large family with severe MR and spastic diplegia which was previously thought to be MASA syndrome, a condition caused by mutations in the *LICAM* gene in distal Xq28. Negative LOD scores with Xq28 markers led to a search for linkage to other regions; this was found in Xp22 where several other XLMR loci are located, although the clinical features of this family differ from that of other XLMR syndromes and conditions in Xp22.

Like MASA syndrome, X-linked hydrocephalus (HSAS) is caused by *LICAM* mutations. Linkage analysis of a Scottish HSAS family, however, indicated that a second locus for the disease was located in Xq27.3, close to FRAXA. Despite this, an *LICAM* mutation was later identified in the proband which segregated with the disease in other family members. The most likely explanation for the discordant linkage data is gonadal and somatic mosaicism in the maternal grandmother of the affected boy.

## ABBREVIATIONS

BSA	bovine serum albumin
CT	computer topography
CVS	chorionic villus sample
dITP	deoxy inosine triphosphate
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EEG	electroencephalogram
EMBL	European Molecular Biology Laboratory
G6PD	glucose 6-phosphate dehydrogenase
HPRT	hypoxanthine phosphoribosyl transferase
kb	kilobase/s
OFC	occipitofrontal circumference
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PVP	polyvinyl pyrrolidone
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulphate
VNTR	variable number of tandem repeats

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# 1. X-linked mental retardation

## 1.1 CLASSIFICATION OF MENTAL RETARDATION

Mental retardation is defined by the World Health Organization as “incomplete or insufficient general development of mental capacities”. The classification of mental retardation assigns a performance index on two criteria; intelligence quotient (IQ) and overall socio-adaptive behaviour, the latter taking into account the individual’s relative performance in school, at work, at home and in the community. Adaptive functioning in people with mental retardation is influenced by personality characteristics, motivation, education, and social and vocational opportunities and may improve with remedial efforts whereas the IQ is likely to remain stable. Mental retardation (MR) is divided into four groups ranging from mild to profound (Table 1), classified according to IQ. This is obtained by assessment with one or more general intelligence tests; the most widely used today are the Stanford-Binet (which tests children from 2 years of age to adolescence) and the Wechsler Intelligence Scale for Children (WISC-R) which is applicable to children in the age range 6 to 16 years.

Classification	Former classification	Stanford-Binet	Wechsler
Mild mental retardation	Dull	52-67	55-69
Moderate mental retardation	Simpleton/feeble-minded	36-51	40-54
Severe mental retardation	Imbecile	20-35	25-39
Profound mental retardation	Idiot	<20	<25

Table 1. Psychological classification of mental retardation (according to the American Association on Mental Deficiency).

## 1.2 HISTORICAL PERSPECTIVE OF X-LINKED MENTAL RETARDATION

The excess of males among mentally retarded individuals was first recognised by Lewis in 1929. He was appointed by the Board of Education to ascertain the extent

of mental retardation in children, so that adequate education and care could be provided for them under The Mental Deficiency Act of 1913 and The Education Act, 1921. At an early stage in the proceedings it became clear that the study should be extended to include mentally retarded adults as well. His three and a half year investigation involved studying children and adults living at home, children in special schools, and residents of mental institutions and covered six different geographical areas in England and Wales. Each of the areas contained a population of about 100,000, (the six areas comprising one-sixtieth of the total population) and represented different industrial groups and social strata. He observed from his study that in children, a) *“the mean incidence of all grades of defect ..... is about 17 per cent higher among boys than girls”* and b) *“the incidence of imbecility and idiocy (severe and profound mental retardation) is about 30 per cent higher for boys than girls, whereas feeble-mindedness (moderate mental retardation) is only 14 per cent higher”*. In adults, he noted that *“the marked sex difference in the incidences of the lower grade defect is again shown by the figures for adults; the mean incidence among males is 33 per cent higher than that for females, whereas the mean incidence of feeble-mindedness is practically the same among men and women”*. He also investigated the family histories of mentally retarded individuals to find out how many of the families had two or more mentally defective individuals and noted that the mother-son, mother-daughter pairs for all grades of mental retardation outnumbered the father-son, father-daughter relationships by more than 2:1. This ratio was 1.5:1 for severe and profound mental retardation. Although he made these observations he confessed that he was unable to offer any satisfactory explanation for the sex differences.

Another survey on the incidence of mental retardation was conducted in 1938 by Penrose who published the results of a 6 year clinical and genetic study of 1,280 patients (710 males and 570 females) who resided in a mental institution in southern England. During this intensive study, he recorded the patient's age, sex, “mental grade”, mental grade of the patient's father and mother, the relationship between the parents, the number of brothers of each mental grade, the number of sisters of each

mental grade, and the clinical diagnosis of the patient. Although his data showed a 26% excess of males in the profoundly mentally retarded and a 14% excess in the severely retarded he stated that “*it is doubtful how far this excess of males among the idiots (profound MR) represents a real excess of male idiots in the general population*” and concluded that “*unequivocal evidence for the significance of sex-linked genes in the causation of mental defect was not forthcoming*”.

A few years later, however, Martin and Bell (1943), accepted “sex-linked” inheritance as the explanation for the presence of severe mental retardation in a family with 11 affected males in 2 generations; theirs was the first report of X-linked inheritance in which mental retardation appeared as the primary manifestation. The mode of transmission was unusual in that all the affected individuals were derived from three sibs: two normal brothers and a normal sister. They suggested that some controlling factor caused suppression of the disease in the normal brothers without affecting their liability to transmit it. There were also two females in the family with “slight mental deficiency” which the authors proposed was caused by an incompletely recessive gene. Seven of the affected males in this family were re-examined in 1981 by Richards et al. and five were shown to have a fragile site in Xq27.3 (two cultures failed). Some of the males had the typical facial appearance of fragile X syndrome and some had macro-orchidism, although no mention of an abnormal physical appearance was made in the original paper of Martin and Bell, apart from one affected man who was described as having a “big face and jaw”. Richards et al., (1981) suggested that the condition should be designated “The Martin-Bell Syndrome”. Since the discovery of mental retardation in association with a fragile site on the X chromosome (Lubs, 1969) other families have been restudied and found to have fragile X syndrome. The family studied by Dunn et al. in 1963, in which the authors concluded that “*the condition is determined by a sex-linked gene of variable penetrance which appears to have entered the family through the phenotypically normal male ancestor*” and that “*the gene may be incompletely recessive causing low-normal intelligence in heterozygous females*” was restudied by Fox et al. in 1980. This family was also found to have fragile X syndrome.

A second family with X-linked mental retardation (XLMR) was reported by Allan et al. in 1944, in which severe mental retardation was present in 24 males in six generations. Affected males appeared normal at birth except for hypotonia but by six months of age, they were unable to hold up their heads which led to the family's description of the patients as "limber neck". They also had spastic paraplegia, ataxia, dysarthria, athetoid movements, muscle hypoplasia and were incapable of speech. Macro-orchidism was not present. At the time of the initial study in 1944, at least 15 women of reproductive age or younger were potentially carriers of this condition. The family was re-investigated in 1990 (Schwartz et al.; Stevenson et al.) when three subsequent generations were added to the pedigree and five additional affected males identified. In total, 29 males were affected in 7 generations. Additional clinical features included scoliosis, pectus excavatum, foot deformities, joint contractures, elongated facies with normal head circumference, bitemporal narrowing, and large, simple ears. In 1990, Schwartz et al. presented linkage data suggesting that the gene for the "Allan-Herndon-Dudley syndrome" (AHDS) is located in Xq21 (peak LOD score obtained with *DXYS1*). Bialer et al. (1992) also studied a large XLMR family with clinical features closely resembling AHDS in which tight linkage to *DXS72* also placed the locus for the disease segregating in their family to Xq21. It appears therefore, that this family also has AHDS. A third family with AHDS has since been ascertained (Schwartz et al., 1994a) and studied using markers located in Xq12-Xq22. Multipoint analysis indicated that the AHDS locus is 0.6 cM distal to *DXS326*. All three families are presently being analysed with all available microsatellite markers between *DXS1111* and *DXS3* in an attempt to further refine the region of localisation of AHDS.

After the paper of Allan et al. (1944), XLMR publications did not appear again until 1961 when Losowsky studied a family with 12 severely mentally retarded members in four generations and 2 less severely affected females. It is suspected that this family, too, had fragile X syndrome although they have since been lost to follow-up (Stevenson et al., 1994). Probably the best known of the early XLMR reports is that of Renpenning et al. (1962) who, as a medical student in Canada, studied a large

family of Dutch Mennonite extraction consisting of 20 affected males with head circumferences at the lower end of the normal range, prominent ears and short stature, in addition to severe mental retardation. Two of the affected males had seizures. This family has been restudied by Fox et al. (1980) and Schwartz et al. (1993) and a locus for Renpenning syndrome has been shown to map to Xp21.1-q12. Renpenning syndrome has, in the past, also been used to describe fragile X syndrome and non-specific XLMR as well as the syndrome described above, but it is evident from the localizations of these conditions and the clinical dissimilarities between them that they are very distinct disorders. Turner et al. (1971) suggested that approximately 10% of all moderately retarded males could have Renpenning syndrome although they accepted that different loci may be involved. In 1969, Snyder and Robinson published a family with nine males with mental retardation over three generations. None of the males had any specific facial characteristics, but they had hypotonia and disequilibrium, and the authors concluded that the family had a non-specific form of XLMR. The family was restudied 23 years later in 1992 (Arena et al., 1994), when clinical and DNA studies were conducted on 17 family members. It now appears that the family has a syndromic rather than a non-specific form of XLMR with affected males exhibiting the following features: mild to moderate mental retardation; large head; wide-based gait; marfanoid habitus; diminished muscle bulk; nasal voice; long thin face and high narrow palate and mild scoliosis. Linkage analysis placed the locus for Snyder-Robinson syndrome at Xp22 with a peak LOD score being obtained with *DXS41*.

The delay in recognising XLMR may have been due to inadequate ascertainment; many of the earlier surveys were carried out in mental institutions where the patients were largely severely mentally retarded, but most XLMR is actually of the “community based” mild-moderate type. The fact that the contribution of X-linked genes to mental retardation is greatest in the mild-moderate categories is shown by the greater recurrence risk for male sibs with mild-moderate mental retardation than with severe-profound mental retardation (Turner et al., 1971, Bunday et al., 1989). Priest et al. (1961) found more males than females in state institutions (although



males are probably more likely to be institutionalized because of uncontrollable or violent behaviour) and found that affected sibs were more often male. A population survey of mental retardation in 1965 (Reed and Reed) found a 49% excess of mentally retarded males and showed that a retarded female with a normal male partner was twice as likely to have retarded children as was a normal female with a retarded partner. Further support for XLMR came from the observation that there was a disproportionate number of families with only retarded boys (Wortis et al., 1966); since then other similar surveys have consistently observed a male excess (Moser and Wolf 1971; Turner and Turner 1974; Laxova et al., 1977; Blomquist et al., 1981; Bunday et al., 1989). To account for this male excess, Lehrke (1972) hypothesised that 25% to 50% of all mental retardation was X-linked whilst others have estimated the proportion to be between 10% and 20% (Turner et al., 1971, Turner, 1982).

### 1.3 PREVALENCE OF XLMR

Herbst and Miller (1980) calculated an incidence of XLMR in British Columbia of 1.83 per 1,000 live male births and a carrier frequency of 2.44 per 1,000 live female births, which corresponds to a mutation rate of  $6.1 \times 10^{-4}$ . Assuming a mutation rate of  $3-9 \times 10^{-5}$  for X-linked loci (taking the mutation rates of  $3.2 \times 10^{-5}$  for haemophilia A and  $9.2 \times 10^{-5}$  for Duchenne muscular dystrophy), and a fitness of zero for affected males, they estimated that 7-19 genes cause non-specific XLMR (using Haldane's equation  $\mu = q(1-f)/3$ , where  $\mu$  is the mutation rate,  $q$  is gene frequency and  $f$  is genetic fitness). This is a similar figure to that obtained by Morton et al. (1977) who estimated that there may be 18 X-linked loci for mental retardation. If the mutation rate was  $3.2 \times 10^{-5}$  per locus, they calculated a possible incidence of XLMR of 1.74 per 1,000 births. Opitz (1986) calculated a prevalence of 1/296 for XLMR for both sexes when fragile X syndrome is included (assuming that 40% of XLMR is fragile X syndrome) making the total incidence of all XLMR at least double that of Down's syndrome (Schwartz, 1993).

## 1.4 CLASSIFICATION OF XLMR

There are currently 127 X-linked conditions in which mental retardation is manifest (Neri et al., 1994). 53 of these have been regionally mapped on the X chromosome, although with the exception of fragile X syndrome, the individual frequency of each of these conditions is relatively low. As the abnormal clinical findings of XLMR conditions are documented, they are reclassified as specific diagnostic entries and assigned a MIM number (McKusick 1994). There are now 80 entries with a MIM number. The number of XLMR conditions has more than tripled since the Fourth International Workshop on X-linked mental retardation in 1989 (Neri et al., 1991). This is primarily due to changes in the inclusion criteria; all X-linked disorders where mental retardation has been reported as a consistent finding are now included in the classification system. XLMR conditions are divided into five classes as shown in Table 1.

Conditions in which mental retardation is the primary manifestation (Class 1 and Class 5) account for 62% of all XLMR; 39 of these have been mapped within the X chromosome using linked markers. As XLMR is clinically and genetically heterogeneous, linkage analyses can only be performed in families which are large enough to produce significant LOD scores ( $>2$ ) by themselves. LOD scores cannot generally be added among families unless there is absolute certainty that the same syndrome is involved. Kerr et al. (1991) have proposed that the definition of non-specific mental retardation (MRX) should be "mental retardation in persons of normal physical appearance, who have no recognisable features apart from a characteristic pedigree". They also recommend that the symbol MRX (introduced by Suthers et al. in 1988) be reserved for gene localisations in families with non-specific XLMR (Class 5 in Table 1). Herbst (1980) proposed that only a pedigree in which there are affected males in two generations or in two maternally related sibships, and in which the presence of a fragile site in Xq27.3 has been excluded, should be classified as MRX. The revised nomenclature for MRX genes allocates a number to every MRX family localized under specific guidelines (Mulley et al., 1992a); the

prerequisite for assignment of an MRX or MRXS symbol is a minimum LOD score or multipoint LOD score of 2 between the MR locus and at least one X chromosome marker. The symbol MRXS was introduced to accommodate syndromal forms of XLMR pending assignment of a specific name, usually that of the author who described the first family.

Class	Total number	Gene mapped	Gene cloned
1. Syndromes i.e. clinically recognizable conditions, based on a characteristic pattern of physical anomalies [designated MRXS, (Davies et al., 1991)]. Listed in Table 3.	57	24	4
2. Dominant disorders	6	4	
3. Metabolic disorders	12		9
4. Neuromuscular disorders i.e. clinically recognisable conditions, based on a characteristic neuromuscular involvement. Listed in Table 4.	30	10	4
5. Non-specific mental retardation i.e. conditions where mental retardation is the only consistent manifestation [designated MRX, (Davies et al. 1991)]. Listed in Table 5.	22	18	1
<b>Total</b>	<b>127</b>	<b>56</b>	<b>18</b>

Table 2. Current status of XLMR conditions. Only conditions in which definite linkage to the X chromosome is demonstrated, and where linkage analysis resulted in a LOD score of 2 or more are included in this table. (Adapted from Neri et al., 1994).

## 1.5 IDENTIFICATION AND MAPPING OF XLMR GENES

Mapping of XLMR loci has progressed rapidly over the last few years with the availability of highly polymorphic microsatellites spanning the X chromosome (Weissenbach et al., 1992; Schlessinger et al., 1993; Gyapay et al., 1994), new non-radioactive techniques and automated gel electrophoresis equipment (Mansfield et al., 1994), and the ability to analyse archival material on deceased family members (Jackson et al., 1991). Several overall maps of the X chromosome have been constructed. The initial Généthon map included 25 X-linked “Afm” microsatellite markers that are now well integrated with other markers in physical or genetic maps



(Weissenbach et al., 1992). The second Génethon map contained 80 Afm markers extending over 166 cM (Gyapay et al., 1994), although there were two large gaps in this map: 13 cM in Xq24-25 and 17 cM in Xq27. Murray et al. (1994a) constructed a genetic linkage map using genotypes available from the CEPH (Centre d'Etude du Polymorphisme Humain) and the CHLC (Cooperative Human Linkage Centre). They analysed 128 dinucleotide repeats in 15 families consisting of 185 individuals and produced three linkage maps at varying degrees of resolution, i.e. of increasing density, but decreasing confidence level. The "skeletal" map (total X chromosome length 143 cM) included 23 loci; the "framework" map contained 35 loci (206 cM); and the "comprehensive" map contained 150 loci (266 cM). The average distance between markers varied from 5.7 cM for the framework map to 6.14 cM for the skeletal map. Fain et al. (1995) used the method of crossover mapping to produce a high density two-dimensional linkage map, by incorporating information from five previously published linkage maps (NIH/CEPH Collaborative Mapping Group, 1992; Buetow et al., 1994; Gyapay et al., 1994; Matise et al., 1994; Murray et al., 1994b). They analysed 243 markers in 40 CEPH families and produced a map with an average distance between markers of 5.4 cM (when ordered by two recombinants) and 3.2 cM if the markers were ordered using information from one recombinant, with an estimated total genetic length of 190 cM for the X chromosome.

Analysis of individual large MRX families using a dense map of highly informative markers may result in a localization within a 10-15 cM interval (Kerr et al., 1992; Mulley et al., 1992b; Hu et al., 1994), although an interval of this size could, theoretically, contain approximately 150-400 genes (Mandel, 1994). An overlap between the genetic intervals containing two MRX families does not imply that the same gene is involved and cannot therefore be used to obtain a more restricted localization. Each family must be treated as a separate disorder until the particular genes are identified. The presence of phenocopies (about 1 or 2% of males are likely to be mentally retarded even though they do not carry the mutation segregating in a particular family) may result in mapping errors in some MRX families. The identification of XLMR genes with a broad linkage based localisation will ultimately

depend on systematic analysis of many affected males for mutations in candidate genes. Mandel (1994) has proposed that a lymphoblastoid cell line and preferably also a fibroblast cell line, on one proband and one obligate carrier female from each XLMR family (both MRX and syndromic forms), should be deposited at a cell repository.

By correlating gene location and clinical findings, it may be possible to diagnose new syndromes in pedigrees which have previously been too small for a linkage based classification alone. However, clinical reports are often incomplete, which makes it difficult to determine whether two syndromes which appear similar are the same or not. As the number and density of XLMR genes increases it will become even more important that reports contain precise clinical information. It has been proposed (Schwartz et al., 1994b) that a minimum clinical dataset be used covering three categories: physical measurements (height, weight, OFC, eye, ear, hand, testicular), other clinical observations (developmental history, IQ, hearing, speech, vision, malformations, minor anomalies, dermatoglyphics, spinal curvature), and neurological signs (gait, reflexes, muscle tone and mass, seizures, involuntary movements, behaviour) and that measurements should be reported as percentiles as well as absolute measurements. In addition, for the non-specific XLMR conditions, some normal males should be subjected to the same clinical investigation to ensure that subtle changes noted in the affected males do not represent unrelated familial characteristics (Schwartz et al., 1992). Females should also be studied to note the presence of manifesting carriers in the family.

This thesis is composed of molecular and linkage studies on eight fragile X syndrome families and three other XLMR families in S.E. Scotland which were referred to the Human Genetics Unit for investigation.

Class 1			
MIM number	Syndrome Name	Locus	Description
301040	ATR-X ( $\alpha$ -thalassemia/mental retardation syndrome)	Xq12-q21.3	Microcephaly, "coarse" face, genital and skeletal abnormalities. HbH inclusions.
303600	Coffin-Lowry	Xp22.2-p22.1	"Coarse face", drumstick phalanges, skeletal abnormalities
305000	Dyskeratosis congenita	Xq28	Skin pigmentation, nail dystrophy, leukoplakia of oral mucosa
305450	FG		Macrocephaly, agenesis of corpus callosum, gastrointestinal abnormalities, deafness
309550	Fragile X	Xq27.3	Macrocephaly, long face, everted ears, macro-orchidism
302000	Hereditary bullous dystrophy	Xq26-q28	Short stature, microcephaly, alopecia, bullous dystrophy, hypogenitalism
309590	Juberg-Marsidi	Xq12-q21	Growth retardation, small genitalia, deafness
309605	Miles (MRXS4)	Xp21.1-q22	Microcephaly, asymmetric face, hypogonadism, joint hypermobility
302350	Nance-Horan	Xp22.3-p21.1	Cataract, microcornea, cone-shaped incisors, supernumery teeth
311300	Otopalato-digital	Xq27-q28	Short stature, hearing loss, cleft palate, characteristic face
309510	Partington (MRXS1)	Xp22.1-p21.3	Dysarthria, dystonic movements of hands, ataxia, seizures
304340	Pettigrew (MRXS5)	Xq26-Xq27.1	Long "course" face, hydrocephalus, hypotonia, spasticity, ataxia, seizures
	Porteous	Xp11.4-Xq13	Short stature, high-pitched voice, high forehead, receding hairline
309610	Prieto (MRXS2)	Xp21.1-Xp11.22	Peculiar face, dental anomalies, sacral dimple, joint dysplasia, epilepsy
309500	Renpenning	Xp21.1-Xq12	Microcephaly, short stature
312870	Simpson-Golabi-Behmel	Xq24-Xq28	Macrosomia, "coarse" face, polydactyly, extra nipples, heart defect.
	Snyder-Robinson	Xp22.3-Xp21.3	Macrocephaly, long thin face, high narrow/cleft palate, asthenic body build, scoliosis, marfanoid habitus
309470	Sutherland (MRXS3)	Xp21.1-Xq22	Microcephaly, short stature, small testes, spastic diplegia
309585	Wilson-Turner (MRXS6)	Xp21.1-Xq22	Obesity, gynecomastia, tapering fingers, emotional lability

Table 3. XLMR syndromes (Class 1). (Reproduced from Neri et al., 1994)

Class 4			
MIM number	Syndrome Name	Locus	Description
309600	Allan-Herndon-Dudley	Xq11.4-q21.3	Severe hypotonia, joint contractures, muscular atrophy.
310490	Cowchock-Fishbeck	Xq13-q21	Motor-sensory neuropathy, deafness
310200	Duchenne muscular dystrophy	Xp21.3-1	Pseudohypertrophic muscular dystrophy
312920	Goldblatt	Xq13-q21.1	Spastic paraplegia, nystagmus, optic atrophy
	Gustavson	Xq25-q26	Optic atrophy, hearing loss, epilepsy, spasticity, restricted joint mobility, early death
307000	HSAS	Xq28	Hydrocephalus, stenosis of the aqueduct of Sylvius
303350	MASA	Xq28	Macrocephaly, aphasia, shuffling gait, adducted thumbs, growth retardation
312900	SPG1	Xq28	Spastic paraplegia, ataxia
	Ionasescu	Xp22.3-1	Motor-sensory neuropathy
304700	Mohr-Tranebjaerg	Xq22	Hearing loss, visual impairment, ataxia, spastic paraplegia
310600	Norrie	Xp11.3	Blindness, hearing loss
	Passos-Bueno	Xp21.2-q21.31	Hypotonicity, incontinence, severe generalised muscle atrophy
312080	Pelizaesus-Merzbacher	Xq21.33-q22	Spasticity, cerebellar ataxia, Parkinsonism
311510	Waisman-Laxova	Xq27.2-q28	Parkinsonism, seizures, apparent basal ganglia degeneration
314580	Wieacker-Wolff	Xq11-q22	Contractures, distal muscular atrophy, dyspraxia of ocular and facial muscles

Table 4. Neuromuscular disorders (Class 4). (Adapted from Neri et al., 1994)

Class 5			
Name	Locus	Description	Reference
MRX1	Xp11.4-q21.31	Mental retardation only	Suthers et al., (1988)
MRX2	Xp22.2-3	Macrocephaly, square face, macro-orchidism, short stature	Arveiler et al., (1988)
MRX3	Xq28	Mental retardation only	Gedeon et al., (1991)
MRX4	Xp11.22-q21.31	Speech delay, learning disability	Arveiler et al., (1988)
MRX5	Xp21.1-q21.3	Hyperactive behaviour, speech delay	Samanns et al., (1991)
MRX6	Xq27	Short stature, “coarse face”, short broad hands	Kondo et al., (1991)
MRX7	Xp11.4-q21.3	Mental retardation only	Jedele et al., (1992)
MRX8	Xp11.3-q21.22	Mental retardation only	Schwartz et al., (1992)
MRX9	Xp21.2-q12	Mental retardation only	Willems et al., (1991)
MRX10	Xp11.4-p21.3	Hypotelorism, large ears	Kerr et al., (1992)
MRX11	Xp11.22-p21.3	Hypotelorism, large ears	Kerr et al., (1992)
MRX12	Xp21.3-q21.1	Hypotelorism, large ears	Kerr et al., (1992)
MRX13	Xp22.3-q21.22	Large ears	Kerr et al., (1992)
MRX17	Xp11-q21.3	Mental retardation only	Gedeon et al., (1994)
MRX18	Xp21.1-p11	Mental retardation only	Gedeon et al., (1994)
MRX19	Xp22.3-p21	Mental retardation only	Donnelly et al., (1994)
MRX21	Xp22.3-p21.1	Mental retardation only	Kozak et al., (1993)
MRX24	Xp22.2-p22.3	Mental retardation only	Martínez et al., (1995)
FRAXE	Xq28	FRAXE, mental retardation only	Knight et al. (1993)

Table 5. Non-specific XLMR conditions (Class 5). (Adapted from Neri et al., 1994)



## **2. X-linked mental retardation presenting as developmental delay; DNA testing for fragile X syndrome.**

### **2.1 INTRODUCTION**

Mental retardation is global delay in the development of cognitive learning. The term global is used to differentiate it from specific learning disorders such as dyslexia and dyspraxia, and cognitive to distinguish between learning with understanding and memory learning. Children with physical features suggestive of syndromes associated with mental retardation may be identified at birth or during early infancy, as in the case of Down syndrome, although these disorders represent only a small percentage of the population of intellectually impaired children. The vast majority are identified because of their failure to meet expectations at certain ages; delayed achievement of developmental milestones is the cardinal feature of mental retardation. Most mentally retarded children develop slowly from birth; children with severe impairment show obvious psychomotor delay in the first year of life, moderately retarded children usually exhibit normal motor development but present with delayed speech and language abilities and mild retardation may not be detected until the child attends school. Sometimes, language milestones are delayed disproportionately in relation to motor development. The usual definition of mental retardation is an IQ of 2 standard deviations or more below the general population mean and relies on clinical validation of the child by standard psychometric tests. A general scale of intelligence consists of a variety of tasks which measure different aspects of intellectual functioning and estimate the child's overall ability, as measured by a total score. Many tests are available; the following are a few of the ones most commonly used in the UK.

**Wechsler Intelligence Scale for Children (WISC) - age range 5 to 15 years.** This scale consists of 12 subtests, divided into verbal and performance subscales. WISC scores are obtained by comparing test performances with those scores achieved by children in the same age group as the patient (within a 3 month range). This scale has been revised (WISC-R) and the age range is now 6-16 years.

**Wechsler Pre-school and Primary Scale of Intelligence (WPPSI) - age range 4 to 6½ years.** This scale is similar to the WISC test, but includes design copying and sentence repetition tests for the younger age range.

**Stanford-Binet Intelligence Scale - age range 2 years to adolescence.** This scale is a later version of one of the oldest and most widely used measures of intelligence.

**Cattell Infant Intelligence Scale - age range 2 to 30 months.** This was developed as a downward extension of the Stanford-Binet test.

In addition to the general intelligence tests, there are also performance and non-language tests, tests for physically handicapped children, blind, and deaf children.

### **2.1.1 Epidemiology of mental retardation**

The distribution of intelligence in the general population is a bell shaped curve with mentally retarded individuals belonging to the lower extreme of the distribution (Figure 1). Approximately 3% of the population has an IQ below 70 and less than 0.5% of the population has moderate or severe mental retardation (IQ roughly 20-50). There are more mentally retarded individuals in the population than would be expected from a normal Gaussian distribution and this is attributed to the presence of two groups of mentally retarded patients making up this 3%. The majority of individuals (2.3%) have IQs between 50 and 70 with no demonstrable pathological disease and the remainder (approximately 0.3% to 0.6%) with IQs less than 50 are mentally retarded due to some pathological factor (Moser et al., 1983).

Because a diagnosis of mental retardation relies on an assessment of adaptive behaviour in addition to IQ, the epidemiology of mental retardation varies with age. The reported incidence of mental retardation increases sharply in the early school years, when children are more likely to be detected as having learning difficulties, and then declines in late adolescence as individuals with borderline or mild retardation blend in to the community.

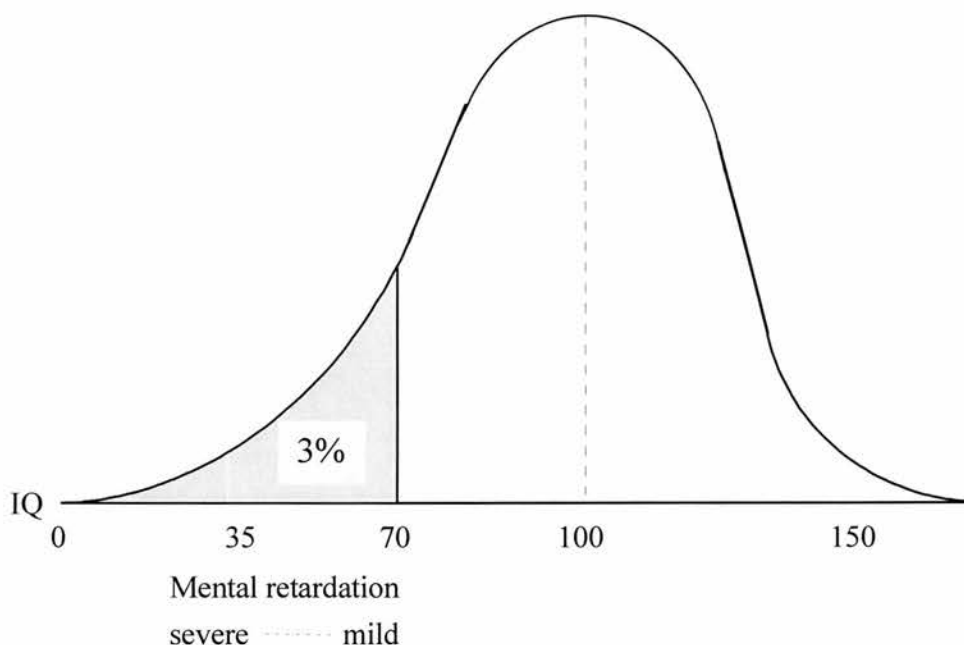


Figure 1. Population distribution of intelligence. (Adapted from Moser et al., 1983) This drawing is not to scale.

### 2.1.2 Etiology and pathogenesis of mental retardation

According to OMIM (Online Mendelian Inheritance in Man) at least 750 genetic disorders include mental retardation as part of the phenotype. An etiological classification groups mental retardation as: **Genetic** - chromosomal and single gene disorders, **multifactorial** - disorders with both genetic and environmental components, and **environmental** - infections, toxins, trauma etc.

### 2.1.3 Fragile X syndrome

Fragile X syndrome is the most common form of familial mental retardation and the second most common cause of mental retardation after Down syndrome (which has an incidence of ~1 in 600 to 1 in 800 live births; Shen et al., 1995). The incidence is 1 in 1,250 males, with a generally milder form of mental retardation occurring in 1 in 2,500 females. Fragile X syndrome constitutes 40% of XLMR (Schwartz, 1993).



### **2.1.3.1 Historical background**

In 1969, Lubs reported a three-generation XLMR family with four affected males who had an X chromosome in which a small part of the tip was separated from the main part of the long arm by a constriction. Four normal female carriers, including the mother of two affected brothers, also had this “marker” X chromosome. Another family with a marker chromosome was described in 1971 (Escalante) although the importance of this observation was not realised until a few years later when two other studies found the constricted X chromosome in 25 males and 1 affected female (Giraud et al., 1976; Harvey et al., 1977). During the next few years, several laboratories were unable to reproduce these findings until Sutherland (1977) demonstrated that the expression of chromosomal fragile sites, including fragile X (*FRAXA*), was dependent on the type of culture medium used, specifically one which was folic acid and thymidine deficient. *FRAXA* was induced in Medium 199 but not in other commercially available media tested. Following this discovery, a series of families with XLMR were tested and many were found to have the fragile site (Turner et al., 1978; Turner et al., 1980). The family investigated by Martin and Bell in 1943, originally reported as a non-specific form of XLMR, was also reinvestigated and shown to have fragile X syndrome (Richards et al., 1981).

### **2.1.3.2 Epidemiology**

Several large surveys have been carried out in order to determine the prevalence of fragile X syndrome. Blomquist et al. (1982;1983) and Gustavson et al. (1986) conducted prevalence studies of mild and severely retarded males in northern Sweden and concluded from their data that approximately 0.1% of the general population would be expected to have a fragile X chromosome. A survey conducted by Webb et al. (1986) in England, found 16 fragile X positive boys among 219 boys with idiopathic mental retardation (1 in 14) and 10 out of 104 mentally retarded girls. When these figures were extrapolated for the population studied, the prevalence of fragile X syndrome became 1 in 1362 males and 1 in 2073 girls, equivalent to an overall prevalence of 1 in 1634 (0.06%). Turner et al. (1986) conducted a similar

survey in New South Wales which resulted in prevalence figures of 1 in 2610 for males and 1 in 4221 for females. Taking these and other studies into consideration, the estimates of the total prevalence of fragile X (mainly from European populations) varied from ~1 in 2500 to 1 in 1100 for males and 1 in 5000 to ~1 in 1650 for females (Webb, 1989). However, all these surveys were cytogenetically based and therefore a proportion (~50%) of affected females will not have been detected (see Cytogenetics, 2.1.3.6). In addition, the contribution of normal transmitting males and mentally normal female carriers to the gene frequency could not be taken into account because a) neither would be detected cytogenetically, and b) the surveys investigated mentally retarded groups only, with extrapolation from their data to give frequencies in the general population.

### **2.1.3.3 Clinical Phenotype**

Fragile X syndrome is characterized clinically by three major features; moderate to severe mental retardation, elongated, narrow facies with large everted ears, and macro-orchidism. Although clinical macro-orchidism ( $\geq 2$  times normal), is uncommon prior to puberty, pre-pubertal boys have significantly larger mean testicular volumes than non fragile X males (Lachiewicz and Dawson, 1994). After the age of 8 there is a sharp increase in the number of fragile X boys with macro-orchidism and it is present in almost 90 percent of post-pubertal males (Kirkilionis et al., 1988). The phenotypic features of affected females tend to be more subtle and in both males and females the phenotype can be extremely variable. The majority of affected males have an IQ between 20 and 50 (Sutherland and Hecht, 1985), although the degree of mental retardation can vary even within families. Additional physical features may be increased head circumference in childhood, coarsening of facial features, hypotonia, and prominence of the forehead and jaw. About 80% of adult fragile X patients have mitral valve prolapse, which is thought to be due to a connective tissue dysplasia. Longevity in fragile X patients appears to be normal; no entity can be exclusively identified as a major cause of death (Partington et al., 1992). No single physical feature is completely diagnostic and many of the features overlap with other mental retardation syndromes; it is often the behavioural

characteristics which alert clinicians to the diagnosis. These include hyperactivity, tics, attention deficits, aggressive outbursts, unusual hand mannerisms such as hand flapping and hand biting, poor eye contact and a reluctance to be touched (Hirst et al., 1992). Many of the behavioural abnormalities diminish as patients enter adulthood. It is difficult to diagnose in newborn babies (apart from greater than average birth weight, male neonates with fragile X syndrome appear normal) and the features slowly accumulate with age.

Only 60% of fragile X males have the three main characteristics and 10% present with mental retardation as the only clinical feature (Hirst et al., 1992). Fragile X syndrome is frequently ascertained through a language learning delay and is therefore generally not suspected before the child reaches 2-3 years of age. Occasionally, however, some fragile X cases are ascertained very early as illustrated by three new cases who were diagnosed below the age of 2 years (discussed later in this chapter).

#### **2.1.3.4 Fragile X syndrome and autism**

Autism occurs in about 5 in 10,000 births with a 3-4:1 male predominance. In 2-3% of families, more than one autistic child is found within the same family. There is much controversy surrounding the issue of whether fragile X syndrome and autism are associated. Einfeld et al. (1989) screened for autistic behaviour in fragile X males and compared this result with the proportion of autism found in non fragile X mentally retarded males. They concluded that the syndromes were not associated. Cohen et al. (1991) however, came to the conclusion that there was an association between the two and estimated that approximately 8% of autistic children have fragile X syndrome and 16% of fragile X males are autistic. To account for the discrepancies between earlier surveys, Einfeld et al. (1989) suggested that clinicians may have been misled into believing that an association existed because two autistic-like behaviours, hand flapping and gaze avoidance occur at a higher rate in fragile X children, compared to other mentally retarded children. Fisch (1993) consolidated all the reports in the literature and proposed the term "concurrence" rather than "association" or "correlation" to describe the relationship between fragile X and

autism. The overall consensus is that fragile X testing is appropriate for any autistic male of undiagnosed etiology (Brown and Jenkins, 1992). 6.1% of males who were referred to our laboratory for fragile X testing had autistic features as part of the phenotype although all were subsequently found to be normal with respect to fragile X syndrome (Figure 12, page 76).

#### **2.1.3.5 Genetics**

The inheritance patterns observed in fragile X syndrome are unique. It is transmitted in an X-linked semi-dominant fashion, with incomplete penetrance. There are several other unusual non-Mendelian properties of fragile X inheritance (Richards and Sutherland, 1992):

- 1) 20% of males who inherit the fragile X mutation are not affected (normal transmitting males, NTMs), but transmit the mutation to their daughters, who are asymptomatic obligate carriers. Mothers of NTMs are normal.
- 2) Daughters of NTMs have a high probability (~40%) that offspring who inherit the abnormal X chromosome will be clinically affected and this risk increases in the next generation to 50%.
- 3) 35% of carrier females are mentally retarded but to a lesser degree than affected males. Affected females receive the fragile X mutation from their mothers, not their fathers.

The observation that the risk of a family member being phenotypically abnormal is dependent upon his or her position in the pedigree is known as the Sherman paradox (Sherman et al., 1985; Opitz 1986); this is illustrated in Figure 2.

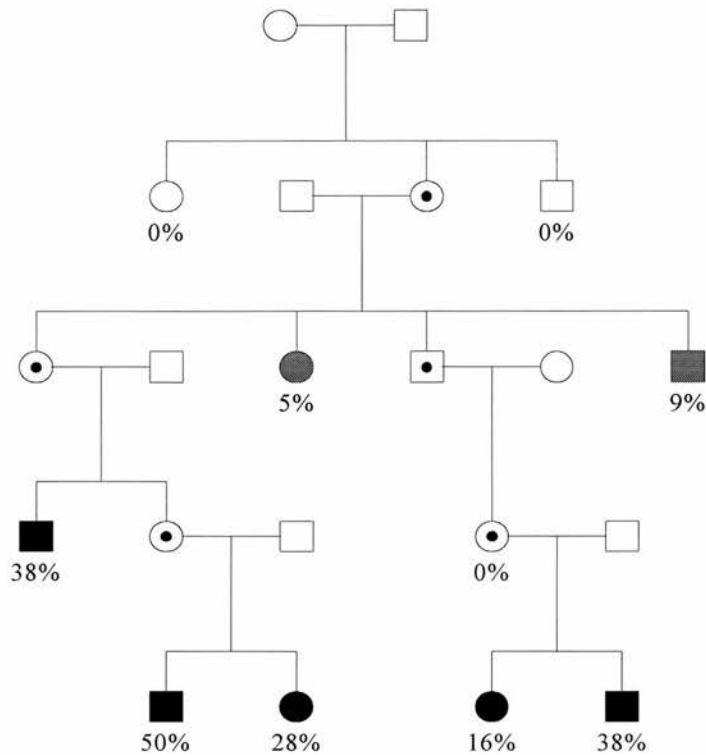


Figure 2. Illustration of the Sherman paradox in a hypothetical family. The figure below each individual is the empirical risk of that person being mentally retarded, according to Sherman et al. (1985). Filled symbols represent mentally retarded individuals (with lighter shading for lower risk), symbols with a dot represent mentally normal carriers.

### 2.1.3.6 Cytogenetics

Fragile X syndrome is named after the characteristic fragile site in Xq27.3 which is detected microscopically as a non-staining constriction in one or both chromatids in metaphase chromosomes. It may also appear as a terminal deletion of Xq28 due to the loss of the acentric fragment, which may be observed elsewhere in the metaphase spread. Cells need to be cultured in medium deficient in folic acid and thymidine, with excess thymidine (Sutherland and Baker, 1986), or in the presence of several additives which disrupt folate metabolism (methotrexate or fluorodeoxyuridine (FUdR)) in order for the fragile site to be induced. These additives must be present during late S phase prior to the mitosis in which expression is observed. All induction conditions appear to result in depletion of supplies of dTTP or dCTP available for DNA synthesis.



More than 87 common (i.e. found in most of the population) and 26 rare (found in less than 1% of the population) other fragile sites have been described (Sutherland and Ledbetter, 1989). Three other fragile sites have now been identified in distal Xq, which could be confused with *FRAXA*. One of these, *FRAXD* (Sutherland and Baker, 1990), is a common fragile site which maps proximal to *FRAXA*, the other two are rare fragile sites: *FRAXE* (600 kb distal to *FRAXA*) and *FRAXF* (more than 1Mb distal to *FRAXA*). Cloning of the *FRAXE* and *FRAXF* regions has revealed, in both cases, the presence of an unstable trinucleotide (CGG) repeat adjacent to a CpG island (Knight et al., 1993; Parrish et al., 1994). *FRAXF* is not associated with any clinical phenotype. *FRAXE* expression, however, has been shown to segregate with a mild form of mental retardation in some families (Flynn et al., 1993) but not in others (Sutherland and Baker, 1992; Allingham-Hawkins and Ray, 1995).

Cytogenetic diagnosis of fragile X syndrome follows specific guidelines for conditions and criteria (Jacky et al., 1991; Dewald et al., 1992). The principal features recommend the use of two or more induction systems, the analysis of at least 50 – 100 cells in males and 75 – 150 cells in females, and the inclusion of a standard chromosomal analysis to exclude other cytogenetic etiologies for the mental retardation. When these guidelines are followed, the sensitivity of detection of *FRAXA* in affected males is quite high, with virtually all affected males expressing the fragile site (in 2% to 60% of cells). However, only 50% of carrier females can be detected in this way, and the cytogenetic test is incapable of detecting carrier males or phenotypically normal carrier females. In addition, the presence of the other fragile sites, *FRAXD*, *FRAXE* and *FRAXF*, indicate that a positive cytogenetic test may not be specific for fragile X syndrome and requires confirmation by molecular testing. The role of cytogenetics in the diagnosis of fragile X syndrome has diminished with the advent of cheaper and more accurate molecular detection and is no longer justified in known fragile X families. Cytogenetic testing is still important, however, among developmentally delayed children referred for fragile X testing, to rule out other chromosomal abnormalities which may be responsible for the child's learning disability. In prenatal diagnosis, there are specific difficulties associated

with cytogenetic detection of the fragile site in chorionic villi and amniocytes with a rate of misdiagnosis of about 5% due to false positives and more frequently, false negatives (Sutherland et al., 1991).

### **2.1.3.7 Isolation of the *FMRI* gene**

#### Genetic linkage studies

The first report of linkage of a DNA marker to fragile X syndrome was a study of the segregation of the Factor IX gene (*F9*; Camerino et al., 1983) in which the disorder was transmitted through a phenotypically normal grandfather. Although no recombinants were identified in the large family described, several families were later reported in which recombination was observed between *F9* and the fragile X locus. Brown et al. (1985; 1988) noted differences between families in which there was tight linkage between *F9* and fragile X, and families which gave negative LOD scores. They suggested genetic heterogeneity as a possible explanation for the discrepancy and subsequently divided their fragile X families into two types, those with and without a normal transmitting male (the existence of non-penetrant males in fragile X syndrome had been recognised since 1985 (Sherman et al.)). Shortly after the *F9* studies were published, three new polymorphic probes were isolated (Figure 3) and reported to be close to the fragile site. These were: i) the highly polymorphic VNTR probe St14 (*DXS52*; Oberlé et al., 1985), ii) 52A (*DXS51*; Davies et al., 1985) and iii) DX13 (*DXS15*; Drayna and White, 1985). Further linkage analysis with these new probes (Brown et al., 1987) in addition to the *F9* probe, was confounded even more when linkage heterogeneity was still evident, irrespective of whether an NTM was present in the family. Heterogeneity tests showed significant linkage heterogeneity on the proximal side of the fragile X locus but not on the distal side. The estimates of genetic distance between *F9* and fragile X varied from 18 cM in the “tightly linked” pedigrees to more than 35 cM in the “loosely linked” pedigrees. New mapping techniques (Fain et al., 1995, and see 1.5) have now established that this distance is 24.5 cM. Following on from the *F9* genetic heterogeneity studies, Suthers et al. (1991a) conducted linkage analysis in 112 fragile X families with five

DNA markers in the interval from *DXS369* proximally to *DXS304* distally, although they failed to find evidence of linkage heterogeneity in the region. The discrepancy between these studies is not fully resolved but is presumably due in part to the difficulties of estimating genetic distances in a syndrome with such unusual inheritance patterns and reduced penetrance.

In the search for the fragile X mutation, many new closely linked polymorphic markers were isolated. Between 1989 and 1991, four new probes were identified which mapped to within approximately 5cM of *FRAXA*: U6.2 (*DXS304*; Dahl et al., 1989a,b), VK21 (*DXS296*; Suthers et al., 1989), RN1 (*DXS369*; Oostra et al., 1991) and VK23 (*DXS293*; Suthers et al., 1991b). Rousseau et al. (1991) isolated three more polymorphic probes: St677 (*DXS463*), 2-35 (*DXS477*) and Do33 (*DXS465*) which map between RN1 (*DXS369*) and the iduronate-2-sulphatase (*IDS*) locus and which are still the closest dimorphic RFLPs to *FRAXA*. The order of these markers is shown in Figure 3.



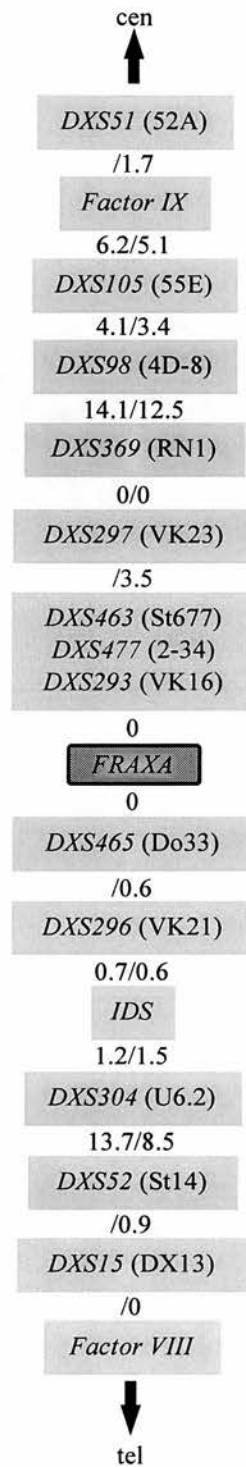


Figure 3. Genetic map of DNA probes in the vicinity of *FRAXA*. Distances between markers are in centiMorgans (cM). Figures on the left are taken from Suthers et al. (1991a,b) and were converted (by the authors) to cM from recombination fractions using the Haldane mapping function. For comparison, figures on the right were obtained from Fain et al. (1995), who used the Kosambi equation.

### Somatic cell hybrids

In 1987, Warren et al. constructed somatic cell hybrids that contained either a human fragile X chromosome or a normal X chromosome in a hamster background. Human lymphoblasts from a fragile X male were fused with Chinese hamster ovary cells deficient in both *HPRT* (Xq26) and *G6PD* (Xq28) activities and the cells cultured under conditions for induction of fragile X expression. By screening for or against *HPRT* or *G6PD* activity, they were able to isolate hybrids containing either exclusively proximal (Xpter - q27.3,  $\mu$ 21D) or exclusively distal (Xq27.3-qter, Q1X) portions of the human fragile X chromosome. The hypothesis was that the fragile site would be a preferential location for translocations between human and hamster material - using a previous observation that chromosomal fragility occasionally resulted in breakage and translocation (Warren and Davidson, 1984). The hybrid panels were shown to have breakpoints within the fragile X site by hybridization with proximal and distal markers (Warren et al., 1990). These hybrids enabled a number of new polymorphic loci to be mapped relative to the fragile site and confined the region containing the fragile X locus to 3 Mb (Suthers et al., 1990; Rousseau et al., 1991; Hirst et al., 1991a).

### Methylation anomalies

The search for cloned DNA containing the fragile X site was not successful until new DNA technology made available the technique of pulsed field gel electrophoresis (PFGE). Areas in which non-methylated sites cluster - CpG islands - are frequently associated with expressed genes and as the sites are relatively infrequent, the fragments generated as a result of enzyme digestion tend to be very large (100-1000kb) and can only be resolved with PFGE (Schwartz and Cantor, 1984). In early 1991, Vincent et al. reported methylation abnormalities in 22 fragile X patients detected by PFGE using the probe Do33 (*DXS465*). Fragile X males showed aberrantly large bands, larger than the 620kb fragment seen in 21 normal males but similar to the large bands seen in female cell lines, suggesting a methylation anomaly in the region. Bell et al. (1991) reported similar results; they observed significant differences in the PFGE pattern after *Bss*HII digestion of DNA from fragile X males

compared to normal male controls. In 19 out of 21 affected males, the normal 600kb *Bss*HII band was either absent or of reduced intensity, consistent with methylation at the *Bss*HII site. An NTM gave a normal pattern, but in 2 affected males, a normal *Bss*HII pattern was also observed. However, the authors failed to give a satisfactory explanation for the discrepancy and suggested that the fragile site could be methylated to an extent not detectable by their assay. It is not clear from the article which criteria were used for classification as “fragile X positive” for inclusion in their study. It is possible that the two males were detected cytogenetically and are therefore false positives, in which case their mental retardation is not due to fragile X syndrome (the authors did point out that these two males had no family history of mental retardation). Alternatively, it is possible that the methylation status in peripheral blood lymphocytes is different to that in other tissues and therefore may not be correlated with mental retardation.

A few months later, further reports on the isolation, cloning and partial characterization of the fragile X mutation were published. These appeared almost simultaneously from groups in France (Oberlé et al., 1991), Australia (Yu et al., 1991) and an American/Dutch collaboration (Verkerk et al., 1991).

#### YAC cloning of the fragile site

Kremer et al. (1991a), constructed a YAC (yeast artificial chromosome) library from DNA from the human/hamster somatic cell hybrid X3000-11 (Nussbaum et al., 1986) which contained a fragile X chromosome from Xq24 to Xqter translocated onto a hamster chromosome. The YACs were screened with DNA probes which map close to the fragile site and XTY26, containing 275 kb of human DNA, was isolated using VK16B3 (*DXS293*). Further analysis revealed that the DNA probes, Do33 (*DXS465*) and 2-34 (*DXS477*) were also present in XTY26, confirming that it contained DNA spanning the fragile site.

YACs crossing the fragile site were also identified simultaneously and independently, in the CEPH library by Mandel (Heitz et al., 1991) and by the

American/Dutch consortium (Verkerk et al., 1991). Regional mapping of YAC clones containing DNA inserts derived from the distal human Xq (Nelson et al., 1991), identified an 80 kb YAC (RS46) which mapped within Xq27.3, proximal to the fragile X associated hybrid breakpoints. However, RS46 did not hybridize to DNA of the distal translocation hybrids and therefore did not cross the breakpoints. A CEPH YAC library was screened using RS46 specific oligonucleotide primers and a 475 kb YAC was identified (209G4) which completely overlapped RS46 and crossed the fragile site. Genetic linkage studies between a dinucleotide CA repeat (*DXS548*) within RS46 and the fragile X locus in families which were previously shown to have crossovers with tightly linked flanking markers, showed that *DXS548* segregated without recombination with the fragile X locus. A CpG island containing five rare restriction enzyme sites was identified 150 kb distal to *DXS548*. This was the same CpG island identified independently by Heitz et al. (1991) and which appeared hypermethylated on fragile X chromosomes (Vincent et al., 1991; Bell et al., 1991).

#### Subcloning and fluorescent *in situ* hybridization

The Mandel group (Heitz et al., 1991) also independently identified the YAC 209G4 by screening with the linked probe St677 (*DXS463*); this YAC was found to contain the proximal probe 2-34 (*DXS477*) in addition to St677 and fluorescent *in situ* hybridization on metaphases from five fragile X patients after induction with fluorodeoxyuridine (FUDR) verified that 209G4 spanned the fragile site. A larger YAC (141H5) containing 520kb of human DNA was isolated by screening with the distal probe Do33; this YAC was also found to contain St677 and 2-34.

Further to the identification of YACs spanning the methylated CpG island, all three groups used cosmid or lambda subclones to walk to the CpG island. Verkerk et al., (1991) constructed a cosmid library from the YAC 209G4 clone and identified a four cosmid contig which spanned the fragile X related CpG island. From one of these cosmids (22.3), a 7.4 kb *EcoRI* fragment was identified which was demonstrated to contain the CpG island and to be a breakpoint cluster region where the majority of

fragile X-associated breakpoints were positioned. The same *EcoRI* fragment, which was equivalent to a 5.1 kb fragment in normal X chromosomes, showed a variable increase in size in fragile X chromosomes when hybridized to *EcoRI* digested DNA from normal and fragile X patients. The normal samples exhibited the expected 5.1 kb fragment, while all the affected fragile X males had larger fragments with variable increases in size. In addition, a) *in situ* hybridization of cloned material from the region mapped precisely within the isochromatid gap characteristic of the fragile site and b) the 5.1 kb fragment contained the *BssHII* site which had been previously demonstrated to be hypermethylated in fragile X chromosomes (Vincent et al. 1991; Bell et al., 1991). The same 5.1kb fragment (designated pfxa1) was also identified by Yu et al. (1991) and from this a 1 kb *PstI* fragment (pfxa2). Similarly, Oberlé et al. (1991) identified three overlapping plasmid subclones (StB12, StX21 and StA22) from YAC 209G4, which covered a 9 kb DNA segment including the CpG island. The 7 kb clone StB12 which mapped to the telomeric side of the CpG island was further cloned after *PstI* digestion and one of the resulting fragments (StB12.3) used as a probe on *EcoRI* digests of DNA from members of fragile X families. This probe also showed increased bands of variable size in affected males, a slightly larger than normal band in normal transmitting males and an increased band in affected females (in addition to the normal band).

All three groups, therefore, had cloned the region encompassing the fragile site and demonstrated differences between normal individuals and affected fragile X patients.

#### **2.1.3.8 Identification of the *FMRI* gene**

Verkerk et al. (1991) used cosmid subclones of YAC 209G4 as hybridization probes to screen a normal human fetal cDNA library. One of these cosmids (c4.1) identified cDNA clone BC22, which was then used to identify related cDNAs from the same library. From overlapping cDNAs, BC72 and BC22, more than 3.7 kb of mRNA sequence and 657 amino acids of encoded protein were deduced. They named the

gene from which the BC22 and BC72 cDNA clones were derived, *FMRI*\* (fragile X mental retardation 1).

At the 5' end of BC72, a repeated DNA sequence was found which consisted of 28 CGG triplets interspersed with 2 AGG triplets [(CGG)<sub>10</sub>AGG(CGG)<sub>9</sub>AGG(CGG)<sub>9</sub>]. Kremer et al. (1991) used PCR to amplify the variable region in the genomic clone pfxa2 and also found that it contained a CGG trinucleotide repeat of 43 copies. However, since pfxa2 was derived from XTY26 which was cloned from a fragile X expressing cell line, it was clear that the CGG repeat is unstable during cloning as well as in fragile X pedigrees. Comparison of pfxa2 with the original cell line, X3000-11, demonstrated that its cloned counterparts (XTY26, λ5 and pfxa1) had lost about 900 bp of sequence during the cloning process. Kremer et al. (1991b) also showed that the CGG repeat region was the site of alterations in fragile X chromosomes and that the somatic cell hybrids μ21D and Q1X had breakpoints within the repeat.

#### **2.1.3.9 Expression studies**

The first indication that the *FMRI* gene was expressed in brain came from the experiments of Verkerk et al. (1991) during the search for *FMRI*. As described earlier (2.1.3.8), the cDNA clone BC22 was identified by screening a cDNA library derived from normal human fetal brain RNA with cosmid subclones of the YAC 209G4. Northern hybridization using BC22 as a probe detected an mRNA of approximately 4.8kb in brain and placenta. Further analysis of other human tissues demonstrated the existence of *FMRI* mRNA in lymphocytes, although it appeared absent in liver, fetal lung, or fetal kidney. In order to determine the normal distribution of gene expression of *FMRI*, Hinds et al. (1993) carried out northern blot analysis on various human tissues; heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas and testis. Brain and testis showed the highest levels, with significant expression in placenta, lung and kidney, and low to negligible levels in

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\* The fragile X mental retardation gene is *FMRI*, the protein product of this gene is FMR1. The mouse homologues are *fmr1* and *fmr1* respectively.



liver, pancreas and skeletal muscle. Multiple transcripts in the region of 1.4 kb were detected in the heart with no signal at 4.4 kb (no explanation is given by Hinds et al. for the discrepancy in transcript lengths: 4.4kb vs. 4.8kb). The existence of multiple species of mRNA may be a result of tissue specific splicing. The existence of moderate levels of expression in human lung and kidney contrasts with the findings of Verkerk et al. 1991, who found negligible levels in fetal lung and fetal kidney. It is unknown whether there is a true variation in expression in these tissues with age or whether the differences are due to experimental artefacts.

#### Variation in level of expression during development

Hinds et al. (1993) also studied expression of the mouse homologue of *FMRI* (*fmr1*) during various stages of development by *in situ* hybridization. They examined whole uterus sections from pregnant mice at various gestations, and found high levels of expression of *fmr1* from day 7 (the earliest stage studied) until day 10 in all embryonic tissues (except bone). After day 10 there was a reduction of the signal and change in distribution, when expression became more heterogeneous between organs, particularly in the brain and liver. There is a marked difference between the level of expression in the embryonic heart (up to day 14) which showed uniformly increased expression, and the adult heart where no signal was detected. A similar study of human fetuses by Abitbol et al. (1993) localised the regions of expression more precisely. They analysed the distribution of *FMRI* mRNA by *in situ* hybridization, in tissue sections from two normal fetuses at 8 and 9 weeks gestation, and from the brains of normal monozygotic twin fetuses at 25 weeks gestation. In the 8 and 9 week fetuses, *FMRI* mRNA was present at a high level in the brain, spinal cord and ganglia, with lower levels in some cartilaginous structures and liver. In the older fetuses, *FMRI* mRNA was restricted to differentiated structures of the brain, especially the hippocampus.

#### Expression of *fmr1* and *FMRI* in testes

Because of the major involvement of human testes in the phenotype of fragile X syndrome, mouse testes were studied at various stages of post-natal development



(Hinds et al., 1993) and at the age of 4 weeks (Bächner et al. 1993), although with conflicting results. Hinds examined testes at 1, 12, 17 and 28 days of age and found that the signal was strong initially (in 1 day old mice) and spread uniformly throughout the testis. Later it appeared to be concentrated in the epithelial cells of the seminiferous tubules. In contrast, Bächner's results demonstrated that the level of *fmr1* expression was 30 to 40 times higher in the spermatogonia than in the surrounding tissues. Devys et al. (1993) used FMR1 specific monoclonal antibodies to ascertain where, in normal human testes, *FMR1* was expressed and found that expression in the cells within the seminiferous tubules was restricted to the spermatogonia only. There was no expression in the spermatocytes or their derivatives and also no expression in the supporting Sertoli cells.

#### Adult expression of *fmr1*

In mouse, high levels of expression were observed in adult brain, ovaries, thymus, oesophagus, and spleen; moderate to low levels in kidney, liver, colon, uterus, thyroid and lung and none at all in adult muscle, heart or aorta. Expression in the adult mouse brain was apparent throughout most of the brain with some specific regions showing higher levels, particularly the hippocampus and cerebellum, with intermediate expression in the cerebral cortex, and minimal expression in the corpus callosum.

#### The mouse as an animal model

Since the expression pattern of *FMR1* at the mRNA and protein level is similar in different tissues of humans and mice (apart from the apparent discrepancy in heart muscle), the mouse may be a good animal model in which to study fragile X syndrome. Transgenic knockout mice have been constructed with an inactivated *fmr1* gene and which show macro-orchidism, learning deficits and hyperactivity (Dutch-Belgian Fragile X Consortium, 1994).

In summary, there is widespread although variable expression of *fmr1* and *FMR1* during early development, after which expression becomes more heterogeneous and in adults is confined to the brain and testes.

#### **2.1.3.10 Expression of *FMR1* in fragile X patients**

Pieretti et al. (1991) studied expression of the *FMR1* gene in lymphoblastoid cell lines and leucocytes derived from patients and normal controls by PCR amplifying from *FMR1* cDNA. They analysed 20 affected males, 10 carrier females and 14 normal controls. Sixteen of the affected males had no detectable expression, all the carrier females showed expression although to different extents and in all the normal controls, expression was observed. The four patients with *FMR1* mRNA showed atypical Southern blot patterns with pE5.1 (*EcoRI*/*Bss*HII double digests); three of these were mosaic for a normal band and a smear of increased size fragments indicating that the *Bss*HII site was not fully methylated. The fourth patient had no normal sized band but showed partial methylation. They concluded that incomplete methylation of the CpG island correlates with the presence of *FMR1* mRNA in fragile X patients and suggested that, in some affected fragile X males, the level of expression of *FMR1* did not reach the necessary threshold to provide normal levels of the protein in the relevant tissues at the appropriate time in development. The results of Pieretti et al. (1991) are in agreement with those of Verheij et al. (1993) who demonstrated complete lack of protein product in lymphoblastoid cell lines from four of five affected males. They did, however, find FMR1 protein in one patient who by DNA analysis was a mosaic for premutation and full mutation size alleles. Verheij et al. (1993) and Ashley et al. (1993) also proved that the CGG repeat is within the 5' untranslated region (UTR) and therefore not translated, and that translation starts at an ATG codon 3' to the CGG repeat.

#### **2.1.3.11 Characteristics of *FMR1* and FMR1.**

The *FMR1* gene consists of 17 exons spanning 38 kb (Eichler et al., 1993), determined both by PCR and restriction analysis, and the direction of transcription is

towards the telomere. The average intron length (2.2kb) and average exon length (112 bp) are similar to those of other vertebrate genes although the first intron is unusually large (9.9kb). Alignment of the predicted protein sequence of human *FMR1* (FMR1) with mouse *fmr1* reveals 97% identity and greater than 98% similarity (Ashley et al., 1993). The largest mRNA variant encodes a protein of 631 amino acids with a molecular weight of approximately 69 KDa, and the smallest encodes a protein of 436 amino acids (Verheij et al., 1995).

#### Alternative splicing in *FMR1*

*FMR1* is subject to alternative splicing at the 3' end of the gene as shown by Verkerk et al. (1993) who used RT-PCR (reverse transcriptase PCR) to study normal transcription of *FMR1* in different tissues. The alternative splicing could theoretically result in 12 distinct mRNA products, each of which could be translated into an FMR1 isoform. The presence of four different proteins has been confirmed by using FMR1 specific antibodies (Verheij et al., 1993). No alternative splicing has been found in the first half of the *FMR1* gene; the CGG repeat is in the same exon at the initiation codon ATG (exon 1) and cannot be spliced out without removing this codon. Two principal modes of alternative splicing have been described for *FMR1* (Verkerk et al., 1993; Ashley et al., 1993); alternative 3' acceptor sites have been demonstrated for exons X, XV, and XVII, while exon skipping can occur with exons XII and XIV.

#### Inter species homology of *FMR1*

The *FMR1* gene is highly conserved among vertebrates, and homologous sequences have been observed in yeast, *Caenorhabditis elegans* and *Drosophila* (Verkerk et al., 1991). The CGG repeat is also found in murine *fmr1* in an equivalent position, except there are fewer repeats (approximately 10) which are relatively invariant among mouse strains (Nelson, 1995a).

#### Similarity of *FMR1* and FMR1 to other genes and proteins

Homology searches of the GenBank, SwissProt and EMBL databases with the *FMR1* cDNA sequence, using the BLAST program, failed to reveal any similarities to

previously cloned characterized cDNAs. However, a short repetitive stretch of amino acids within FMR1 consisting of 19 amino acids with 42% identity and 63% overall similarity was identified. One repeat is contained entirely within exon VIII while the other spans exons IX and X. These repeats were shown by Siomi et al. (1993) to have strong similarity to KH domains which are evolutionary conserved domains found only in RNA binding proteins. Also identified within the carboxy terminal portion of FMR1 was an RGG box (Arg-Gly-Gly) which is also implicated in RNA binding and DNA binding, suggesting involvement of FMR1 in RNA metabolism. Khandjian et al. (1996) investigated the subcellular localization of FMR1 (which had previously been shown to be a cytoplasmic protein (Devys et al., 1993)) and demonstrated that during ultracentrifugation of cytoplasmic fractions of HeLa cells on a sucrose gradient, FMR1 cosedimented with polyribosomes. When the polyribosomes were dissociated with EDTA and subjected to further ultracentrifugation, FMR1 was found to be associated with the 60S large ribosomal subunit. Immunofluorescence studies using monoclonal antibodies directed against FMR1 also showed colocalization with ribosomes. From these data the authors postulated that absence of FMR1 might affect the translational mechanism or alter the normal rate of ribosomal assembly.

#### **2.1.3.12 The CGG repeat in fragile X syndrome**

The CGG repeat is highly polymorphic; normal X chromosomes have repeat sizes ranging from 6 to 54 repeats with a mode of 30 repeats (Fu et al., 1991; Snow et al., 1993). Within this normal range, alleles are stably inherited in families (Fu et al., 1991; Kremer et al., 1991b). Normal transmitting males and their carrier daughters, have CGG repeat lengths of 54-200 repeats (premutations); individuals with alleles in this range show a non-penetrant phenotype and in these cases the CpG island is non-methylated. Affected individuals have CGG repeat numbers greater than 200, and often in excess of 1000 repeats (full mutations). Expansion of the CGG repeat is also accompanied by methylation of the CpG island and cytogenetic expression of the fragile site (Oberlé et al., 1991; Verkerk et al., 1991; Fu et al., 1991). The estimated number of repeats required for a phenotypic effect is approximately 230

although methylation at the CpG island appears to be the critical factor in determining the phenotype. The expanded allele in these patients frequently exhibits somatic variation, resulting in either multiple fragments or a heterogeneous smear of fragments on a Southern blot.

Although the number of repeats in the premutation range is somatically stable within the individual, it is highly unstable upon transmission from generation to generation, with expansion to a full mutation occurring only after transmission through the female germline (see **2.1.3.16**). Some rare cases of reduction in size have been observed (Oberlé et al., 1991; Yu et al., 1991). When the premutation is transmitted from father to daughter the repeat either remains the same size or increases slightly, and recent evidence has shown that decreases in repeat length between father and daughter may occur quite frequently (Fisch et al., 1995). The degree of expansion from premutation to full mutation may depend upon the sex of the child, according to Loesch et al. (1995) who found that the difference in size between the mother's premutation and the child's full mutation was greater in boys than girls.

### **2.1.3.13 Founder effects in fragile X syndrome**

Since affected males rarely reproduce, a very high mutation rate has been proposed to maintain the frequency of the disease (Brown, 1990). Haldane (1935) had shown that for X-linked genes, a deleterious allele can be maintained in the population such that the proportion of sporadic cases  $m = su/(2u + v)$  where  $s$  is the selection coefficient in males and  $u$  and  $v$  are the male and female mutation rates. As fragile X males almost never reproduce,  $s = 1$ , therefore one-third of all cases should be *de novo* assuming equal mutation rates in both sexes. However, new mutations, in which expansion of the CGG repeat is responsible for the phenotype, have not been found and in all fragile X families where grandparents of probands were studied, one grandparent was found to carry the mutation (Macpherson et al., 1994). Therefore, carriers of mutant alleles must be present in the general population (Smits et al., 1992). Initial evidence that a founder effect was involved in fragile X came from linkage disequilibrium studies (Richards et al., 1992) using the dinucleotide repeats



*FRAXAC1* and *FRAXAC2* which closely flank *FMRI* (Richards et al., 1991). Three haplotypes which accounted for only 15% of normal chromosomes were found in 58% of fragile X chromosomes. Similar studies by Hirst et al. (1993) using *FRAXAC1*, Macpherson et al. (1994) using *FRAXAC1*, *FRAXAC2*, and *DXS548*, and Oudet et al. (1993) with *FRAXAC2* and *DXS548* found a pronounced difference in the distribution of flanking haplotype between fragile X and normal chromosomes. Snow et al. (1994) carried out further haplotype analysis using *DXS548* and demonstrated enrichment of the 194 bp allele among fragile X alleles and depletion of the 196 bp allele. The 204 bp allele, although not enriched on fragile X chromosomes, showed a significant association with a number of repeats greater than 40. There was a high frequency of association of the most prevalent repeat number (30) with the *DXS548* 194 bp allele.

#### **2.1.3.14 Structure of the CGG repeat**

Sequence analysis of the *FMRI* CGG repeat (Snow et al., 1994) has shown that it is a composite array with sets of CGG repeats interspersed by regularly spaced AGG triplets. Sequencing across the repeat in four different cohorts: normal chromosomes; premutation alleles; full mutation carriers and individuals with a possible diagnosis of fragile X syndrome, showed that the majority of alleles contained at least one AGG interruption within the CGG repeat. A typical array of 29 repeats had the structure (CGG)<sub>9</sub>AGG(CGG)<sub>9</sub>AGG(CGG)<sub>9</sub> (designated 9.A.9.A.9). Among the normal alleles ( $\leq 52$  repeats), 43 contained 1 AGG, 74 had 2 AGGs, 8 had 3 AGGs and 5 alleles were pure CGG repeats with no interspersed AGGs. The frequency of uninterrupted CGG repeats was higher in the premutation allele sample (8 alleles out of 14 had no interruption) and in fragile X chromosomes, 3 alleles out of 5 had AGG interruptions. For the latter, the authors concluded that the sequence in these 5 cases was obtained from a subpopulation of cells containing a premutation length allele, which was too faint to be detected on a Southern blot (the technical difficulties of PCR amplifying and sequencing across the repeat in full mutations precludes study of the nature of the repeat in these patients). Hirst et al. (1994) sequenced across the *FMRI* repeat in 102 normal males and found that 96 of

these consisted of short CGG tracts interrupted approximately every tenth repeat by an AGG triplet with a core unit of [AGG(CGG)<sub>9</sub>] added at the 3' end. They identified five classes of array (types 0-4): type 0 arrays are non-interrupted repeats and make up 6% of chromosomes; type 1 have one intervening AGG repeat with a range in size of 16-36 repeats and make up 28% of chromosomes; type 2 have two AGGs, a range of 28-45 repeats and are the most common form, being found on 64% of chromosomes. Types 3 and 4 (three and four intervening repeats respectively) were rare making up less than 1% of chromosomes. Sequencing of the CGG repeat also demonstrated that pure CGG repeats occur predominantly at the 3' end suggesting polarity for the loss of AGG interruptions (Eichler et al., 1995); this has also been observed to be the case in the occurrence of new mutations in several human minisatellite repeats (Jeffreys et al., 1994). Sequences with no AGG or with the configurations 9.A.≥20, 10.A.≥20 and 11.A.≥20 may be predisposing alleles or fragile X "protomutations" (Snow et al., 1994). Approximately 3-5% of the population carry uninterrupted arrays with greater than 20 CGG triplets, either alone or at the 3' end of the array. The data presented by Snow et al. (1994) indicate that there may be an overlap range within which repeats may be stably or unstably inherited.

#### **2.1.3.15 Mechanism and models of CGG expansion**

Instability of the CGG repeat is thought to be the result of slipped strand mispairing during replication with expansion of the repeat region in repeat unit increments (Snow et al., 1994), and pure CGG repeats being more prone to replication slippage. This can be demonstrated *in vitro* as the presence of more shadow bands on denaturing gels containing PCR products using primers flanking the repeat, assuming that the shadow bands arise as a result of slippage of *Taq* polymerase during PCR (Hauge and Litt, 1993). Comparison of the length of these arrays suggests that pure CGG tracts may have arisen by the loss of the interspersed AGG triplets possibly by deletion during replication slippage. The generation of uninterrupted arrays may be a continuous and dynamic process by which precursor fragile X arrays can arise from within the normal population (Hirst et al., 1994).



Several models have been proposed to explain the behaviour of the fragile X mutation. The model of Morton and Macpherson (1992) postulated that four types of allele exist at the fragile X locus. Normal alleles (N) mutate at a rate of  $2.5 \times 10^{-4}$  to stable alleles (S) which have 50 or more CGG repeats. S alleles are converted to unstable alleles (Z) at a rate of about 1.1% per generation and the full mutation allele (L) only arises from Z during female meiosis but at a rate of 74% per generation. The pool of mutation chromosomes, which is constantly being lost from the population due the decreased reproductive fitness of affected individuals, could therefore be continually renewed from the normal population. If fragile X syndrome is currently in population equilibrium, then a fairly large pool of mutant alleles would be required to maintain the disease frequency. More recently, Ashley and Sherman (1995) proposed a multistep/multipathway model which took into account both meiotic and post-zygotic expansion. The mutation rate  $\mu$  was taken as the rate at which an unstable allele arises from a stable allele was assumed to be equal in males and females. Meiotic expansions were assumed to occur equally in oogenesis and spermatogenesis, while mitotic expansions were limited to the maternally inherited X chromosome. The model also assumed that decreases in size are not true reductions but are due to lack of mitotic expansion in paternally derived alleles.

### 2.1.3.16 Timing of expansion

It is not clear when amplification of an unstable repeat occurs although evidence is mounting that the transition from premutation to full mutation occurs only after transmission through the female germline, and then only for a short period during early embryogenesis.

Fragile X affected fetuses are mosaic with respect to repeat number and following the establishment of mosaicism the repeat becomes stably transmitted in somatic tissues. Wöhrle et al. (1993) showed that somatic variation of CGG repeat length is based on a mosaic of cells with different but stable *FMR1* alleles. They cloned fibroblasts from two affected individuals; a 13 week old positive male fetus, and an adult male patient, both of whom showed typical somatic variation. Dilution plating of the cells resulted in isolated colonies, each carrying only one particular repeat size. After an estimated 22 rounds of DNA replication, the fragment lengths had not changed. Another 13 week old affected male fetus revealed almost identical patterns of repeat lengths in DNA isolated from different tissues indicating that mosaicism had already been established and stabilized at this time of development. Wöhrle et al. (1993) concluded that if expansion of a premutated repeat does not start before the formation of a zygote, parental imprinting must exist in the *FMR1* region to allow for the distinction between paternally and maternally derived premutations. They suggested that carrier parents transmit a premutated *FMR1* allele to offspring and that large expansion to a full mutation takes place in a particular window of early development, exclusively on maternally derived X chromosomes, giving rise to a mosaic of cells carrying alleles with different expansions of the repeat.

Further evidence for post-conceptual expansion came from analysis of sperm from four affected males (Reyniers et al., 1993). Sperm DNA exhibited only premutation alleles while somatic cells (peripheral blood lymphocytes and Sertoli cells from a testicular biopsy) displayed a full mutation. From these results, the authors postulated that either the transition from premutation to full mutation does not occur

in the gametogenic precursors or there is selection against the full mutation due to a requirement for *FMR1* expression in spermatogonia and oogonia.

Additional evidence for the early establishment of somatic mosaicism has come from a prenatal diagnosis carried out in our laboratory (2.3.1.2). Both male fetuses in a monozygotic twin pregnancy were found to carry the full mutation. DNA analysis after termination of the pregnancy revealed identical patterns in the CVS and in all fetal tissues examined. Because the twins were monozygotic, the twinning event occurred sometime between the third and the eighth day of development, suggesting that, in this case, the somatic mosaicism was also established within this period.

Devys et al. (1992) obtained similar results when they studied two pairs of monozygotic twins who had identical band patterns on Southern blotting. The twins in their study were monochorionic and monoamniotic which is a later form of twinning occurring between day 10-20. The timing of CGG expansion can be deduced from the data presented by Devys et al. (1992) to be within day 5 and day 20 of development. If expansion only occurs mitotically then it must take place sometime after day 5 when the gametogenic and somatic cell lineages separate and before day 20 when the late form of twinning suggests that mosaicism is completed.

Although it has been established that males with a full mutation pass only a premutation size allele to their daughters, it has not been proven conclusively (and would obviously be very difficult to do so) that a full mutation carrier mother transmits only a premutation sized allele which then undergoes post-zygotic expansion, to her offspring. When a premutation is transmitted from father to daughter, the allele can either remain unchanged in size, become smaller or become slightly larger, although an allele which changes in size remains in the premutation range. We studied a fragile X syndrome family (discussed in 2.3.3.3) in which a normal transmitting male passed on premutation alleles which were successively smaller in each of his four obligate carrier daughters (Figure 25), the eldest daughter being 17 years older than the youngest. The difference between the largest and smallest allele was estimated to be 20 repeats. Two possibilities could account for

this observation: a) the NTM's sperm carried highly heterogeneous premutation alleles and the reduction in size with time is a chance effect or b) there is continual selection against sperm with large premutations during spermatogenesis. The NTM was deceased at the time of DNA analysis on his offspring and therefore the size of his premutation is unknown.

There is, as yet, no evidence which determines whether changes in premutation size from father to daughter occur during or after meiosis; this might be resolved by single sperm analysis. If separate spermatozoa were shown to carry different sized premutation alleles it would suggest that expansion takes place during meiosis. This has been demonstrated to be the case in spinocerebellar ataxia (*SCA1*), which is caused by expansions in an unstable CAG trinucleotide repeat in the ataxin-1 gene. Chong et al. (1995) analysed the *SCA1* repeat in blood and sperm of two affected individuals. Single sperm were separated using a micromanipulator and peripheral blood leucocyte DNA was diluted out until approximately 2-5 genome equivalents remained; the single sperm DNA and low copy leucocyte DNA were then PCR amplified using nested PCR with primers flanking the CAG repeat. The results showed that the expanded alleles in sperm were more heterogeneous than the expanded alleles in blood. The normal allele in each case showed complete homogeneity (apart from one sperm which the authors attributed to sample contamination). To date, a study of this nature has not been carried out on sperm from males with a fragile X premutation.

An alternative possibility to account for the observations in fragile X syndrome is that somatic instability results from multiple independent transitions from pre- to full mutation in many different embryonic cell lineages, resulting in mosaicism which is unrelated to somatic instability of the full mutation itself. The currently available experimental data cannot distinguish these possibilities.

### 2.1.3.17 Methylation and X-inactivation in fragile X syndrome

When the *FMRI* CGG repeat expands to approximately 230 repeats or more, the entire 5' region of the gene becomes abnormally methylated. The methylation of CpG residues extends from nucleotide position +71 (relative to the start of transcription), includes the CpG island, the repeat itself, to just beyond the *XhoI* site distal to the repeat (Hornstra et al., 1993). The effect of methylation is the abolition of *FMRI* transcription, concurrent absence of FMR1, and expression of the fragile X phenotype. Normal transmitting males are unmethylated in the 5' region of *FMRI*.

The methylation pattern on the normal inactive X chromosome in females is equivalent to that found in males with a full mutation (Kirchgnessner et al., 1995). Carriers of the full mutation display methylation on both the inactive and the mutation bearing X chromosomes, while premutation carrying females are methylated only on the inactive X chromosome. In normal females, there appears to be a range of X-inactivation ratios around the mean of 50:50, ratios of 60:40 and 70:30 are common and 10% of normal females have ratios of 80:20. In fragile X syndrome, non-random X-inactivation alone cannot account for the fact that only 35% of females with a full mutation are mentally impaired, unless tissue specific skewing of X inactivation occurs in the brain. Non-random X-inactivation may account for the variability in severity and the observation that females are usually less severely mentally retarded than affected males. It is also possible that a threshold level of FMR1 is required for normal brain function. Monozygotic female twins have been described in which one twin was intellectually normal and the other was retarded, although both were heterozygous for the full mutation (Kruyer et al., 1994) and both expressed the fragile site in 7% of cells. In the normal twin, the X chromosome carrying the fragile X mutation was the active chromosome in only 30% of cells but in the retarded twin it was active in 85% of cells, suggesting that, in this case, X-inactivation and mental retardation are correlated.

The patterns of methylation at the CpG island of *FMRI* are variable between embryonic and extra-embryonic tissues. In contrast to expansion of the CGG repeat



between day 5 and day 20 of embryogenesis, methylation in CV tissue usually does not occur until sometime after the 10th week of gestation. The majority of chorionic villus samples have been shown to be undermethylated or unmethylated at the CpG island, whereas tissues from affected fetuses only 6 weeks old were methylated in this region (Luo et al., 1993). Sutcliffe et al. (1992) also demonstrated methylation differences between chorionic villi derived from a male fetus with a full mutation and tissues from the aborted fetus; the CV sample was unmethylated and the fetal tissues were methylated. Devys et al. (1992), however, found that the abnormal methylation patterns characteristic of the full mutation were present in 8 of 9 chorionic villi from affected fetuses, while chorionic villi from 6 out of 7 normal females were unmethylated at the CpG island on both chromosomes. The 7th case had patterns equivalent to those seen in normal females, although the authors could not rule out the possibility of incomplete digestion for the methylation sensitive enzyme. The CpG islands of all genes except those which are active during embryogenesis (and excluding the primordial germ cell genes which are demethylated in the third trimester) undergo total demethylation at the morula stage, followed by gene specific methylation at the time of implantation of the blastocyst (at 1-2 weeks gestation). In the mouse, CpG islands in extra-embryonic tissues are known to escape this global *de novo* methylation which is in accordance with the observation that methylation in human CVS tissue is not always comparable to fetal tissues.

Evidence provided by three groups have indicated that it is not the expansion of the CGG repeat which is responsible for the fragile X phenotype but the concomitant methylation of the CpG island (McConkie-Rosell et al., 1993; Rousseau et al., 1994; Smeets et al., 1995). Two phenotypically normal brothers with a full mutation, in addition to cytogenetic expression of *FRAXA* (6% and 30%) were described by Smeets et al. (1995). Methylation analysis showed that a mentally retarded member of the family (a grandson of one of the brothers) was a mosaic for a methylated full mutation and an unmethylated premutation, whereas the two normal brothers had unmethylated full mutations. The type of mosaicism seen in the affected patient was very unusual in that the heterogeneity was shown by a “ladder” of bands ranging



from premutation range to full mutation. Levels of mRNA were normal in the two brothers and absent in the affected boy. These findings have important implications for prenatal diagnosis because methylation in CV tissue is not a reliable indicator of methylation in the fetus and the size of CGG expansion is often used alone as a predictor of intellectual outcome in a male fetus.

#### **2.1.3.18 Fragile X syndrome not due to CGG amplification**

Fragile X syndrome is an atypical genetic disease in that the vast majority (probably more than 99%) of patients have a mutation in one specific region of the gene rather than a variety of mutations (point mutations, deletions, rearrangements etc) throughout the entire gene. However, there is a small group of patients who have the classic phenotype, without expression of the fragile site, and without amplification of the CGG repeat. These patients have mutations which, with one exception (De Boulle et al., 1993) result in complete absence of FMR1 protein. The mutations are summarised in Table 6 and the extents of the deletions are depicted in Figure 4.

Author	Phenotype/ family history	Type of mutation/deletion	Extent of deletion/ nature of mutation	No.
Gedeon et al., 1992a	Clinical features of fragile X syndrome	Inherited deletion	Entire <i>FMR1</i> gene and 2.5Mb flanking DNA.	1
Wöhrle et al., 1992b	25 year old male with MR but no family history. Fragile X phenotype.	<i>De novo</i> deletion	Deletion of <250kb includes CpG island, CGG repeat and 3 proximal exons	2
De Boule et al., 1993	27 year old male with extremely severe fragile X phenotype	<i>De novo</i> point mutation	Ile367Asn	3
Tarleton et al., 1993	23 month baby with developmental delay	<i>De novo</i> deletion	Deletion spans at least 3Mb and removes <i>FMR1</i> plus flanking DNA.	4
Gu et al., 1994	16 month boy with developmental delay and fragile X phenotype	<i>De novo</i> deletion	Exons 1-7 and ~80kb of proximal sequence deleted	5
Trottier et al., 1994	23 year old male with MR and typical fragile X phenotype	Deletion (mother not analysed)	Approx 70-100kb 5' of the repeat to near exon 9	6
Meijer et al., 1994	Family history of MR	Inherited deletion	Approx 1.4kb 5' of the CpG island to the beginning of the CGG repeat (total 1.6kb)	7
Hirst et al., 1995	8 year old boy with MR	<i>De novo</i> deletion	660bp including <i>FMR1</i> promoter, CpG island and transcription initiation site	8
Hirst et al., 1995	25 year old male with moderate MR and fragile X phenotype.	<i>De novo</i> deletion	Deletion from proximal to <i>FRAXAC1</i> to beyond exon 10.	9
Lugenbeel et al., 1995	3 year old boy with developmental delay	<i>De novo</i> point mutation	Deletion of adenosine at position 373 (Thr125) of <i>FMR1</i> mRNA.	10
Lugenbeel et al., 1995	Male patient with fragile X phenotype. Family history of MR.	Inherited point mutation	GG to TA change at position 23714-23715 in the genomic sequence.	11
Prior et al., 1995	18 month old baby with delayed speech and walking.	Inherited deletion (gonadal mosaicism in mother)	At least 5.2kb (the region encompassed by pE5.1)	12
Quan et al., 1995a	6 year old boy with MR plus some atypical features of fragile X syndrome	Inherited deletion	9Mb from <i>DXS105</i> to ~160-500kb 3' of the CpG island	13
Quan et al., 1995b	18 year old boy with MR and cherubism	<i>De novo</i> deletion	Mosaic for deletion of 8.7kb including exon 1, and a full mutation	14
De Graaff et al., 1995	Four unrelated fragile X males	<i>De novo</i> deletions (in 2 patients, others unknown)	Mosaics for deletion of CGG (varying from 150-600bp) and flanking sequences, and full mutation.	15

Table 6. Summary of point mutations and deletions which involve *FMR1*. Numbers in the last column refer to Figure 4.

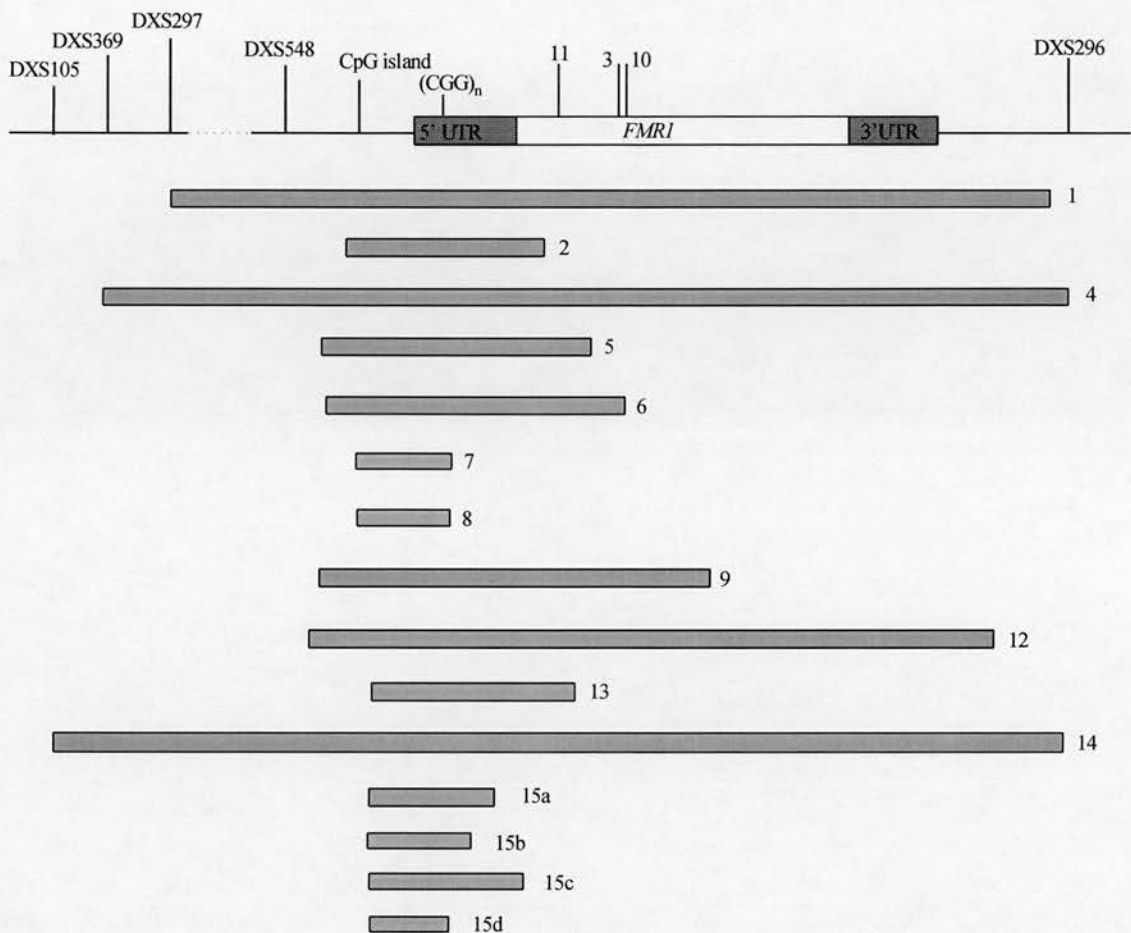


Figure 4. Approximate locations of deletions and point mutations in the region of *FMRI*. Numbers refer to Table 6. Not to scale.

The deletions and point mutations summarised in Table 6 and illustrated in Figure 4 were all found by Southern blotting using probes which detect expansions in the CGG repeat at the 5' end of the gene. It is likely therefore, that other deletions and point mutations may exist in other parts of the gene, which have yet to be detected. The existence of deletions and point mutations in the 5' region of *FMRI* which do not involve expansion of the CGG repeat, but result in the fragile X phenotype, have established that fragile X is a single gene disorder.

The first point mutation of *FMR1* (No. 3 in Table 6) was found in a patient with the fragile X phenotype, no cytogenetic expression of *FRAXA* and a normal CGG repeat length (De Boulle et al., 1993). This patient had a particularly severe phenotype with an IQ of less than 20, dysmorphism, macro-orchidism, and spastic paraparesis. He was an affected member of a family with X-linked glycogenosis (XLG) due to phosphorylase kinase deficiency although the symptoms of this disorder (hepatomegaly, growth retardation, hypercholesterolaemia) diminish after adolescence. Mental retardation is not a feature of XLG and none of the other family members were mentally retarded (or had macro-orchidism). This increased severity of the phenotype in this patient suggests that abnormal FMR1 is more deleterious than complete absence of the protein.

One of the point mutations (No. 10 in Table 6) described by Lugenbeel et al. (1995) resulted in a frameshift and predicted a premature stop codon 66 amino acids 3' to the deletion. An mRNA transcript was detected (as indicated by reverse transcriptase PCR) but Western blot analysis was unable to identify a protein product of the expected size. The two base pair change (No. 11 in Table 6) was a splicing mutation which resulted in two mRNA transcripts of reduced size (one as a result of splicing out exon 2 and the other was created by splicing out exons 2 and 3). Even though splicing out of exons 2 and 3 does not alter the reading frame and therefore a protein of reduced size could be generated, Western blot analysis failed to detect any protein. The authors concluded that these two patients provide strong evidence that absence of FMR1 results in the fragile X phenotype.

The 1.6 kb deletion (No. 7 in Table 6) reported by Meijer et al. (1994) and the deletion/expansion mosaics (No. 15 in Table 6) reported by De Graaff et al. (1995) may have arisen by similar mechanisms with expansion of an unstable repeat being a predisposing factor for a deletion. Studies of deletions in other human disorders have shown that the deleted regions are flanked by short repeats which are presumed to undergo replication slippage resulting in the formation of a loop between the repeats, which is subsequently excised. A small region 5' of the CGG repeat has been

proposed by De Graaff et al. (1995) to be a deletion hotspot; the deletions in the four patients they described all had 5' breakpoints within 30 bp of each other. Although Figure 4 is not drawn to scale, there is a suggestion that this may be the case.

#### **2.1.3.19 Molecular diagnosis of fragile X syndrome**

During the search for the *FMR1* gene, numerous probes spanning the CGG repeat and the CpG island were developed from YACs containing the fragile site. Most of the probes which are currently in use in diagnostic laboratories were derived from the 5.2kb *EcoRI* fragment isolated from the cosmid subclones BC22 and BC72 (2.1.3.8). The fundamental test for fragile X syndrome is the measurement of the length of the CGG repeat, often in conjunction with analysis of the methylation status of the *FMR1* CpG island. This can be achieved by:

- a) PCR amplification across the repeat
- b) Southern blot analysis with DNA probes
- c) Southern blot analysis of PCR products

PCR amplification across the repeat. The first PCR-based method for assessment of length variation within the CGG repeat was developed by Fu et al. (1991) using primers **c** and **f** as illustrated in Figure 5. This is a reliable method for amplifying across the repeat in normal individuals although there are two major limitations to all methods involving PCR amplification (discussed below). In their cohort of 492 X chromosomes Fu et al. (1991) found that the overall heterozygote frequency was 63% with the most frequent allele (found in 30% of chromosomes) estimated at 29 repeats by comparison with size markers. Brown et al. (1993) sequenced across the repeat and established that the most common allele actually consists of 30 repeats, the high CG content of the repeat presumably caused mobility differences during electrophoresis.



## LIMITATIONS OF THE PCR TEST

- 1) **Failure of the full mutation to amplify.** The high GC content of the repeat makes it impossible to amplify (and sequence) across full mutation alleles and large premutation alleles.
- 2) **Preferential amplification in heterozygote females.** This is defined as a difference in the yield of amplified product in heterozygotes so that the larger allele is always much weaker than the smaller one. It is generally related to the size difference between alleles and therefore the premutation allele of a carrier female is often difficult to detect. In addition, the premutation allele appears as an indistinct band with a spread of about 10 repeats, presumably as a consequence of *Taq* polymerase slippage.

In order to improve the efficiency of the PCR assay, many groups have experimented with different primers, cycling parameters, buffers (pH and components), other additives (DMSO, glycerol, detergents), dGTP analogues (dTTP, 7-deaza-dGTP), nested PCR (Levinson et al., 1994), and various DNA polymerases including *Pfu* (Chong et al., 1994). However, no group has yet been able to amplify across the full mutation. One approach which may have potential is an adaptation of the minisatellite variant repeat (MVR) PCR (Jeffreys et al., 1991). In this method a forward primer is situated outside the repeat and a reverse primer consisting of a string of repeats (which would be (GCC)<sub>6</sub> for the fragile X repeat), and a non-homologous "tail" is situated so that it anneals within the repeat itself. The reaction is driven by a third primer which is complementary to the tail only. The tail is designed so that it will not anneal anywhere in the genome. In theory, there should be product from every repeat so that a ladder is produced with the final peak representing the most distal repeat unit (depending on the orientation of the primers). This method has worked successfully for amplification of the variant repeat in *DIS8* (Yamamoto et al., 1994) and the CAG repeat in myotonic dystrophy (Warner et al., in preparation) and it is likely to prove useful in the other triplet repeat disorders. Initial experiments with the *FMRI* CGG repeat have not been successful although



this may be due to the interspersed AGGs and the high GC content of the repeat (CGG/GCC repeats are responsible for the phenotype in only two of the triplet repeats disorders identified so far, fragile X and *FRAXE* mental retardation).

Southern blot analysis with DNA probes. Southern blotting analysis remains the most reliable method for detection of an increase in repeat size. It is simple, relatively inexpensive (through wise choice of enzymes), usually unambiguous and has the additional advantage of providing a simultaneous assay for methylation status at the CpG island. However, the time from receipt of blood to final result can be up to 10 days. Four segments of DNA derived from the 5.2 kb *EcoRI* fragment, from which *FMR1* was isolated, are available as DNA probes (Figure 5):

- a) StB12.3 and pfxa7 are 1.2kb *PstI* fragments derived from either YAC 209G4 or XTY26 (Oberlé et al., 1991; Kremer et al. 1991a). pP2 is a 1.2kb *PstI* fragment derived from pE5.1 (Oostra and Verkerk, 1992). These probes detect a normal band of 5.2kb with *EcoRI* digests in addition to an unmethylated fragment at 2.8kb in a double digest (e.g. *EcoRI* + *EagI*, *EcoRI* + *BssHII*). If *HindIII* is used instead of *EcoRI*, the methylated fragment is just under 5.0 kb and the unmethylated fragment is approximately 2.5kb (Figure 5).
- b) pE5.1 and pfxa1 are the same 5.2kb *EcoRI* fragment isolated from YACs 209G4 and XTY26 respectively (Verkerk et al., 1991; Yu et al. 1991). They span the CGG repeat and the CpG island and detect a normal band of 5.2kb on *EcoRI* digested DNA. In a double digest of *EcoRI* and *EagI*, three bands are detected; the methylated X gives a band at 5.2kb and on the unmethylated X chromosome, this is cut into fragments of 2.4 and 2.8kb.
- c) Ox1.9 is a 1.9 kb *BamHI-EcoRI* fragment (Nakahori et al. 1991) subcloned from a cosmid derived from YAC141H5. Ox1.9 detects the same fragments as StB12.3, pfxa7 and pP2.
- d) pfxa3 and Ox0.55 are essentially the same fragment (~500 bp) which lie immediately distal to the CGG repeat. pfxa3 is a *PstI/NheI* fragment subcloned



from the 1.06kb *Pst*I fragment spanning the CGG repeat and the CpG island (Yu et al. 1991). Ox0.55 is generated by PCR amplification (Nakahori et al. 1991). Hybridization with these probes results in a band of approximately 1kb on *Pst*I digests of DNA from normal chromosomes.

With the exception of the probes in d) which detect a full mutation as a smear greater than ~1.6kb, all the probes detect a full mutation as a band greater than ~5.7kb. Full mutations can appear as a discrete band of increased size, or as several bands, or as heterogeneous smear. In about 12% of affected males (some reports state 15-20%) and 6% of full mutation carrying females, a band is seen in the premutation range as well as in the full mutation range (Rousseau et al., 1994). These individuals are mosaics who have both the non-methylated premutation and methylated full mutation in their leucocytes.

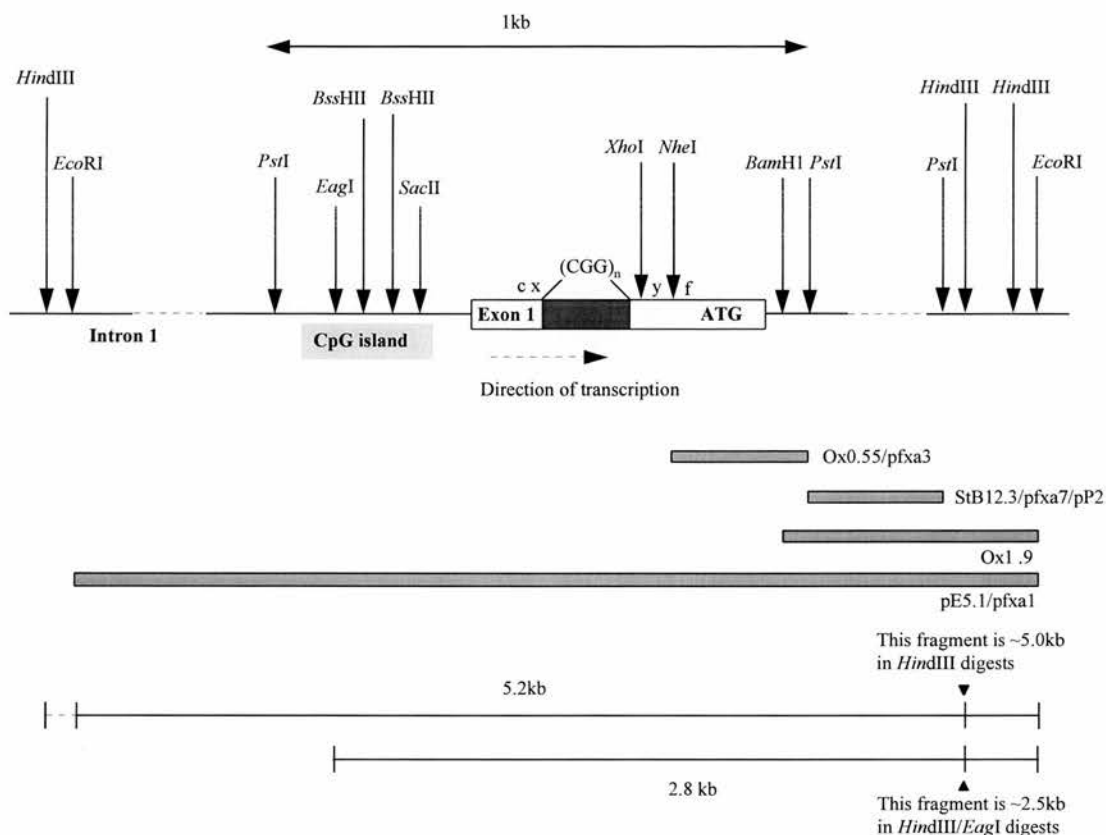


Figure 5. Diagram of *FMRI* in the region of the CGG repeat, showing restriction enzyme sites and DNA probes used in the diagnosis of fragile X syndrome. PCR primers c and f (Fu et al., 1991) and x and y are indicated. Not to scale.

### Southern blot analysis of PCR products

A combination of PCR and Southern blotting was developed by Pergolizzi et al. (1992) as a rapid method of identifying full mutations. PCR amplification was carried out using primers which closely flank the repeat, the PCR products were transferred to a nylon membrane and hybridized with a  $(CGG)_5$  probe. I tested this method but with variable success. As expected, there was preferential amplification of the normal allele in carrier females and some full mutation males still failed to amplify. Male premutation alleles, however, amplified fairly well. The advantages of this method over a traditional Southern/alkali blot are: a) results can be obtained

within 2 days, b) it is cheap, and c) it can be carried out on DNA from mouthwash samples. However, the sensitivity is not as high as with the Southern/alkali blot.

#### **2.1.3.20 Prenatal diagnosis of fragile X syndrome**

Since the discovery of the nature of the mutation causing fragile X syndrome, prenatal diagnosis by direct DNA analysis of chorionic villi, is technically a relatively simple procedure. Currently, in our laboratory, for each prenatal diagnosis, the following are carried out:

- a) Determination of fetal sex using primers at the pseudoautosomal boundary locus (*PAB*; Ellis et al., 1990) or the amelogenin locus (*AMEL*; Bailey et al., 1992).
- b) PCR across the repeat using either primers **c** and **f** (Fu et al. 1991 and see **6.1.4.1**), or **x** and **y** (**2.2.1.2**) with detection of products by denaturing polyacrylamide gel electrophoresis.
- c) Southern/alkali blotting using both *Hind*III digests and *Hind*III/*Eag*I double digests irrespective of the sex of the fetus. The reason for this is that a premutation in a male appears in the 2.7-3.1kb region of the gel where the resolution is greater.

In certain circumstances, a closely linked CA repeat, such as *DXS548* (Riggins et al., 1992, *FRAXAC1* or *FRAXAC2* (Richards et al., 1991), may be useful.

The possible outcomes can be summarised as follows:

Sex of fetus	PCR result	Southern blot result ( <i>Hind</i> III digest)	Southern blot result ( <i>Hind</i> III/ <i>Eag</i> I digest)	Predicted outcome
M	Allele in normal range	Fragment in normal range (~5.0kb)	Fragment in normal range (~2.5kb)	Normal
M	No product	Fragment in normal range (~5.0kb)	Fragment in normal range (~2.5kb)	Normal
M	No product or faint smear in premutation range	Fragment in premutation range (>5.2kb). No sign of smear in full mutation range	Fragment in premutation range (>2.7kb). No sign of smear in full mutation range	<u>Probably</u> NTM (see text)
M	No product	No normal fragment of ~5.0kb. Band or smear above 5.6kb	No normal fragment of ~2.5kb. Band or smear above 5.6kb	Male with full mutation
F	Two alleles in normal range	Fragment in normal range (~5.0kb)	Fragments in normal range (~5.0kb and ~2.5kb)	Normal
F	One allele in normal range	Fragment in normal range (~5.0kb)	Fragments in normal range (~5.0kb and ~2.5kb)	Normal
F	One allele in normal range	Fragment in premutation range (>5.2kb) in addition to normal fragment of ~5.0kb	Fragments in premutation range (>2.5kb and >5.2kb) in addition to normal fragments of ~2.5kb and ~5.0kb	Premutation carrier
F	One allele in normal range	Fragment of ~5.0kb in addition to smear >5.6kb	Fragments of ~2.5kb and ~5.0kb in addition to smear >5.6kb	Full mutation carrier

Table 7. Summary of possible outcomes of prenatal diagnoses.

The two outcomes which may give problems in the interpretation of the results occur when a premutation carrier male or a full mutation carrier female are predicted. In the former case, the male carrier could potentially be a mosaic with a large proportion of cells having an unmethylated premutation in *FMR1* and the remainder, a methylated full mutation which is too faint and/or heterogeneous to be detected on

a Southern blot. In addition, methylation at the CpG island cannot be used to distinguish a large unmethylated premutation and a small full mutation because the methylation status in CVS tissue is not equivalent to that in the corresponding fetal tissue (as discussed previously in **2.1.3.17**). A prenatal diagnosis carried out in our laboratory which resulted in this dilemma is described in **2.3.3.3**.

When a full mutation carrier female is diagnosed, it is not possible to predict the intellectual outcome, either from the size of the expansion, methylation at the CpG island, or by measurement of the degree of non-random X-inactivation. This is a genetic counselling issue which may arise frequently in the context of prenatal screening.

#### **2.1.3.21 Prenatal and population screening for fragile X syndrome**

Whether the time is right for prenatal screening of fragile X syndrome is a contentious issue [Moore, (1992); Palomaki and Haddow, (1993); Bonthron and Strain, (1993); Bunday and Norman, (1993); Howard-Peebles et al., (1993); Palomaki, (1994)] which has not yet been resolved in the UK. Fragile X syndrome appears to be an obvious candidate for population-based prenatal screening for the following reasons: it is a relatively common genetic disorder; the phenotype may be severe; no treatment is available; and the new mutation rate (of a mother with alleles in the normal range producing a child with full mutation) is probably nil. In a pilot study in Canada, Rousseau et al. (1993) analysed DNA from 10,624 women and found that 30 (1 in 354) carried a premutation sized allele. A later study by Rousseau et al. (1994) found 3 premutation alleles among 164 couples suggesting that as much as 0.9% of the general population may carry a premutation (although these 3 alleles were found in male spouses and the sample number was small). The prevalence of affected individuals (1 in 1,250 males and 1 in 2,500 in females) is similar to the prevalence of cystic fibrosis for which prenatal screening programmes have been in place in Edinburgh (Mennie et al., 1992) and other UK centres for several years. As fragile X syndrome is an X-linked condition (even though it is semi-dominant,



affected males do not produce affected daughters) only pregnant women need to be tested and therefore the partner can be ignored.

Population and prenatal screening is presently only being carried out for a few genetic disorders worldwide; for  $\beta$ -thalassaemia in some Mediterranean countries, Tay-Sachs disease in Ashkenazi Jews in the USA, and sickle cell anaemia in certain high risk racial groups in the USA. The effect of these programmes has been a reduction in the birth incidence, sometimes as much as 95% in the case of  $\beta$ -thalassaemia in Cyprus. In Edinburgh, prenatal screening of cystic fibrosis (CF) has resulted in a two thirds decrease in the number of babies born with the disease.

However, a prenatal screening programme for fragile X syndrome would probably not have such a dramatic effect on birth incidence because of the characteristics of the disorder, such as the difficulty in predicting the degree of mental retardation in a female fetus with a full mutation. In addition, since premutation alleles are thought to be derived from large normal alleles, the gene pool of successive generations will continually be added to (unlike the gene pool of CF mutations). The actual effect of a prenatal screening programme for fragile X syndrome will only be shown by a pilot study.

Screening a non-pregnant population would be an alternative method of detecting carriers of fragile X in the population. This could be done in the neonatal period or in school children, although there are ethical considerations involved in testing children if the aim is to reduce the birth incidence of the disease in the next generation, rather than to detect it in the present generation. Children under 16 can consent to genetic testing only if they are able to understand why they are being tested and the significance of the result (Clarke et al., 1994). If they cannot, (which may be the case in fragile X full mutation children) then the parents have the right to decide for the child until he or she becomes 18. For this reason, testing for fragile X is limited to testing through GPs or in antenatal clinics at the time of routine maternal serological tests. There are advantages and disadvantages to both approaches. Screening during pregnancy is known to create anxiety for the couple and also puts

pressure on the laboratory carrying out the tests, although the uptake rates for testing during pregnancy are higher than GP based screening.

### Practical considerations

The nature of the repeat precludes amplification of large premutations and full mutations by PCR, therefore it is impossible to distinguish a normal female who is homozygous for two alleles of the same repeat number, from a carrier female with a full mutation or large premutation. A female who has two clearly distinguishable alleles in the normal range however, could be excluded from further testing, but a female who shows only one discernible allele must proceed to Southern blotting. The proportion which must be analysed further could be as high as 45%. The reasons for this are discussed in 2.3.2.2.

#### **2.1.3.22 Testing developmentally delayed children for fragile X syndrome**

Fragile X testing is recommended for any child with mental retardation of unknown etiology. The cytogenetic test has now been replaced by the more reliable DNA test in most genetic centres in the UK, although the cytogenetic test is still necessary to rule out any other chromosomal abnormality which may be a cause of the mental retardation.

It is important to establish the cause of a child's learning or behavioural disability for several reasons. Firstly, if a diagnosis of fragile X syndrome is made, then there are implications for other family members, and relatives may request carrier testing or prenatal diagnosis. Second, for some parents, it is important to be able to name the condition responsible for their child's mental retardation. Third, a definite diagnosis of fragile X syndrome may indicate that special schooling would be appropriate, which could be beneficial, both for the child and for the rest of the family.

#### **2.1.4 Objectives of this study.**

Prior to 1991, trinucleotide repeat expansion mutations were an unknown form of mutation, but since the characterization of the fragile X mutation, eleven triplet expansions (at ten loci) have been found to be associated with a specific disease and/or fragile site (Willems, 1994). The repeat varies between diseases but generally the neurodegenerative disorders (Huntington disease, SCA1, X-linked spino bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and the allelic Haw River syndrome, and Machado-Joseph syndrome) have repeats consisting of CAG triplets, while the syndromes associated with fragile sites have CGG or CCG triplets. Recently, Friedreich's ataxia was found to be caused by a GAA triplet repeat expansion in an intron of the frataxin gene (Campuzano et al., 1996). The GAA repeat is polymorphic on normal chromosomes and is also unstable upon transmission, although Friedreich's ataxia is unusual in that it is the only disease caused by a triplet repeat expansion in which the inheritance is autosomal recessive.

The identification of the molecular basis of fragile X syndrome has clarified some of the previously puzzling genetic features of the disorder and has also had a major impact on laboratory diagnosis of the syndrome with most genetic centres currently providing a DNA based service for carrier testing and prenatal diagnosis.

The aims of the study presented in this chapter were four-fold: a) to determine the proportion of pre-school age children whose mental retardation was due to fragile X syndrome, b) to provide a molecular fragile X test for older children and adults with mental retardation for confirmation of diagnosis, c) to examine the possibility that polymorphic variation of CGG repeat number is correlated with IQ, and d) to address important practical issues concerning the nature and timing of repeat expansions during transmission in a prenatal diagnostic situation.

## 2.2 METHODS

### 2.2.1 Optimization of methods for DNA testing

Since the identification of the nature of the mutation causing the majority of fragile X cases, many genotyping protocols have been published. As there are several different classes of mutation, all affecting the same region of *FMRI*, a good diagnostic test must be able to identify each type, with high specificity (proportion of unaffected individuals who have a negative result on testing) and high sensitivity (proportion of true positive results). After five years evaluation (since the identification of *FMRI*) of several different methods in many different laboratories, the consensus appears to be that Southern blotting with a double digest is the most reliable test with PCR being a useful, though not essential, adjunct. Some approaches are better than others; my experiences with Southern blotting and PCR are as follows.

#### 2.2.1.1 Southern blotting

The choice of restriction enzyme and probe is determined by the diagnostic information required. The most popular enzyme combinations in the literature are *EcoRI* and *EagI* or *EcoRI* and *BssHII*, although other methylation sensitive enzymes such as *BanI* and *SacII* were used in early reports. I found *HindIII* and *EagI* to be the best combination because they both digest DNA in *HindIII* buffer (50mM Tris-HCl, pH 8.3, 50mM NaCl, 10mM MgCl<sub>2</sub>, 1mM DTT) at 37°C. *BstZ1*, however, has an incubation temperature of 50°C, which means that double digests of *BstZ1* and either *HindIII* or *EcoRI* have to be carried out in two steps. I have had relatively few problems with incomplete digestion using the *HindIII/EagI* combination. In addition, the low-medium salt composition of the *HindIII* buffer allows good gel resolution. The most popular probes in published reports appear to be StB12.3, Ox1.9 and pE5.1. I have had comparable results with the first two (we do not have pE5.1 in the laboratory) but preferentially use StB12.3 because it is a “cleaner” probe (which may be important in detecting weak heterogeneous full mutation smears in females). I have found the following probe/enzyme combinations to be the most useful in our laboratory in the following situations:

**For routine screening of mentally retarded patients.**

In males: a single digest of *HindIII* or *EcoRI* and hybridization with StB12.3 or Ox1.9.

In females: a single digest as above, in addition to a double digest of *HindIII* or *EcoRI* and *EagI* or *BstZI*. These two digests are run side by side on the agarose gel for comparison.

A typical Southern blot obtained in the testing of mentally retarded individuals referred for fragile X testing is shown in Figure 9 (page 73).

**For detection of the full mutation in an individual in a known fragile X family**

As above, although in certain circumstances a single *BglII* digest is useful as the normal fragment is approximately 12kb - this has the effect of condensing a very diffuse full mutation. In females carriers of a full mutation, a double digest will determine the length of the CGG repeat as well as the proportion of active/inactive X chromosomes carrying the mutation. A double digest in a male would detect "methylation mosaics" who have a band in the unmethylated premutation range as well as a methylated band/smear in the full mutation range. I would not recommend the *PstI*/pfxa3 or Ox0.55 combination for detection of a full mutation because these probes give a normal fragment at approximately 1kb which means that a full mutation is spread out and may be very difficult to detect, and in my experience this probe also gives a lot of background signal.

**For detection of the premutation in an individual in a known fragile X family**

The smaller probes Ox0.55 and pfxa3 are useful for detecting smaller premutation alleles in *PstI* digested DNA because the separation between alleles is greater. However, *PstI* digests do not provide information about the methylation status of *FMRI* which is important in distinguishing a large unmethylated premutation and a small methylated full mutation. In this study the best combination has been found to be *HindIII*/*EagI* digests in both males and females and hybridization with Ox1.9 or StB12.3. With this enzyme/probe combination, a premutation appears in the



2.7-3.1kb region of the gel where the resolution is almost as good as in the 1kb region with *Pst*I digests and Ox0.55 or pfxa3 as a probe.

### 2.2.1.2 PCR across the CGG repeat

The advantages of the PCR test are that the number of CGG repeats can be determined accurately and smaller amounts of DNA template are required. However, DNA testing for fragile X by PCR across the CGG repeat is not straightforward. There are several reasons for this: a) the high GC content of the repeat makes it impossible to amplify full mutations; b) PCR additives (such as 7-deaza-dGTP) whilst improving efficiency, reduce visibility of products after ethidium bromide (EtBr) staining (by hindering intercalation of EtBr between DNA strands); c) the inherent “slippage” properties of DNA polymerases which produce shadow bands can make interpretation difficult. Another drawback of PCR is that the methylation status of the CpG island cannot be assessed. This is necessary to distinguish large unmethylated premutations and small methylated full mutations. The first PCR method published for the *FMRI* CGG repeat (Fu et al., 1991) used primers **c** and **f** (Figure 5 and 6.1.4.1) which flank the repeat and which gave a product of 308 bp for an allele of 30 repeats. I tried this method and as it worked fairly well, continued to use it routinely. A typical autoradiograph obtained in routine PCR testing for fragile X syndrome is shown in Figure 10 (page 74).

In order to improve the efficiency of the PCR, particularly the yield of premutation alleles, I made modifications to the PCR protocol by:

- i) Adding 10% glycerol
- ii) Increasing the amount of DNA template to 250-500 ng
- iii) Increasing the number of cycles to 35

These changes had the effect of slightly increasing the yield, and the addition of glycerol reduced the lane background.

PCRs were carried out with the addition of  $\alpha$ -<sup>32</sup>P-dCTP (2 $\mu$ Ci to each reaction).

Later, I adapted the radioactive method so that PCR products could be analysed using



the automated laser fluorescence sequencer (A.L.F., Pharmacia). This involved using a fluorescent primer (either forward or reverse) in the PCR reaction. One advantage of this non-radioactive method, is that since only one DNA strand is being labelled, scoring of alleles should be more reliable. However, the sensitivity of detection is not as high as with the radioactive method and I found that premutation alleles (particularly in females) appeared extremely weak because of the problems of preferential amplification of the smaller allele. For this reason, I reverted to the radioactive method. Another advantage of using  $\alpha$ -<sup>32</sup>P-dCTP is that if a premutation allele appears very faint on an autoradiograph, the same dried gel can be exposed to another piece of X-ray film for a longer period. An increased amount of PCR product can be run on another A.L.F. gel but the volume of reaction has to be limited 10 $\mu$ l or less. Even though I chose the internal radioactive labelling method for routine PCR testing, it became apparent later that there were pros and cons for both methods (discussed in 2.3.2.2).

In a further attempt to improve the efficiency of PCR across the repeat, I designed primers **x** and **y** which, according to the base composition, should have been more compatible with each other than **c** and **f**. After a limited number of trials it was found that they offered no advantage over primers **c** and **f** in terms of yield of premutation alleles. However, they were useful in that they resulted in a smaller product and therefore required a shorter electrophoresis run on PAGE. The sequences which were obtained from the EMBL sequence, HSFxDNA, the 5.2kb *Eco*RI fragment isolated by Verkerk et al. (1991) and Kremer et al. (1991b) are:

**x:** 5' - TCA CTT CCG GTG GGA GGG CCG CCT CT - 3'  
**y:** 5' - AGA GGT GGG CTG CGG GCG CTC GA - 3'

An allele of 30 repeats gives a product of 221bp and amplifies under the same conditions as the primers **c** and **f**.

### **2.2.2 Fragile X testing for mental retardation**

Fragile X testing in a mentally retarded individual is a simple determination of the size of the repeat; the presence of a full mutation is diagnostic of the disorder. DNA

testing of blood samples received from patients with unspecified mental retardation was carried out using both Southern blotting (2.2.1.1 and 6.1) and PCR (2.2.1.2 and 6.1). Figures 9 and 10 show typical Southern blot and PCR autoradiographs (pages 73 and 74).

#### **2.2.2.1 Testing developmentally delayed children for fragile X syndrome**

A cytogenetically based screening programme to detect fragile X syndrome in mentally retarded children has been operating in New South Wales since 1984 (Turner et al., 1992). Other screening programmes have been implemented in New York State (Nolin et al., 1992) and south east Spain (Gabarrón et al., 1992), although the latter studies involved screening mentally retarded males only. The proportion of fragile X cases identified were 3.5% of females and 10.1% of males in the Australian study, 9% in New York State, and 14% in the Spanish study.

In order to determine the proportion of fragile X cases in S.E. Scotland, as detected by DNA methods, a programme for testing mentally retarded children was initiated in October 1991. General practitioners, community paediatricians and hospital paediatricians in Lothian, Fife and the Border regions were informed that this service was available for all cases of mental retardation of unknown etiology. The requirements for the test were at least 3 mls of EDTA anticoagulated blood for extraction of DNA and if possible a separate lithium heparin blood sample sent directly to the Cytogenetics Laboratory for identification of the fragile site. Clinicians were requested to enclose with the specimen, a form detailing the clinical presentation, family history if any, degree of developmental delay, dysmorphism if present, whether a duplicate sample had been sent for cytogenetic testing, and if a previous cytogenetic analysis had been carried out.

#### **2.2.2.2 Testing for fragile X syndrome in adolescents and adults with mental retardation**

Although the initial strategy was to test children with developmental delay (targetting children under the age of 5½ years), blood samples were also received from older

children and adults for confirmation of diagnosis. These made up a substantial proportion of the total number of samples received (50.5%). A small number of patients were referred from adult mental institutions in Lothian and Fife.

### **2.2.3 Analysis of the *FMRI* CGG repeat in three intellectually different populations; correlation of repeat number with IQ**

As described previously, the distribution of intelligence in the general population is an asymmetric bell shaped curve (Figure 1, page 16), with mentally retarded individuals making up 3% of the area under this curve at the lower extreme of the distribution. Patients with fragile X syndrome (males with a full mutation and penetrant full mutation carrier females) fit into this 3% although the IQ range for these patients is highly variable. *FMRI* premutation alleles are thought to be derived from large normal alleles at the upper end of the normal CGG repeat range, which, after several generations have lost one or more of their interspersed AGG triplets and become pure and unstable CGG repeat alleles (most normal alleles have two AGG interruptions, most premutations have no interruptions, Zhong et al., 1995).

#### Do males with premutations have any phenotypic features of fragile X syndrome?

Although males with a premutation are generally considered to be clinically unaffected, recent studies have suggested that normal transmitting males may have mild features of fragile X such as behavioural problems (Dorn et al., 1994) and some characteristic clinical features (Loesch et al., 1994). Hagerman et al. (1996) described three males who presented with learning and behaviour problems who had some characteristic clinical features of fragile X and *FMRI* expansions in the premutation range. Two of the three boys had a small degree of *FMRI* methylation and all three had reduced levels of FMR1 protein in lymphoblastoid cell lines (ranging from 10% to 35% of normal). Rousseau et al. (1994) have shown that 12% of males with a premutation were mentally retarded (5% with borderline MR, 5% with mild MR, and 2% with moderate MR). In premutation carrying females, the total was 5% (3.4% with borderline MR, 0.8% with moderate MR, and 0.8% with severe MR), although the authors stated that these figures were not significantly

elevated when compared to the control groups (4% for males and 3% for females). The most likely explanation for these observations is tissue specific mosaicism with unmethylated premutations being present in lymphocytes and methylated full mutations present in neural tissue.

#### Allelic association with IQ.

In the Quantitative Trait Loci (QTL) Project, Plomin et al. (1994) attempted to identify allelic association (linkage disequilibrium) with IQ using DNA markers in or near genes likely to be relevant to neural functioning. They analysed 46 diallelic markers, 14 microsatellite repeats (2, 3 and 4 bp repeats) and one 48 bp repeat on DNA from 278 pairs of non-identical twins in the top and bottom 5% of the intelligence distribution (the extremes of the IQ distribution were used so that more markers could be analysed on smaller numbers of individuals). In the first report of this ongoing study, associations were found with 5 out of 46 diallelic markers and with 3 of the trinucleotide markers, one of which was the *FMRI* CGG repeat. For each marker, the frequency of the most common allele was compared to the combined frequency of all the other alleles, then the next most common allele was compared to all other alleles, and so on until a frequency of 0.20 was reached. For the *FMRI* CGG repeat, the frequency of the most common allele (30 repeats) was 0.59 in the low IQ group, and 0.32 in the high IQ group. However, when they repeated the experiments on another group of twins in the top and bottom 1% of the population, some of the results were not reproducible. The authors admitted that statistical analysis of multiallelic markers is problematic because of the polymorphic nature of these repeats. One interesting observation from their study is a difference between IQ and allelic frequency with the marker CTGB33 (although it was not a statistically significant difference); this is a triplet repeat which was found by screening a human DNA library and which is expressed in brain (Li et al., 1993). One of the most notable (and reproducible) examples of this phenomenon of allelic association is that of the apolipoprotein E4 allele and late onset Alzheimer's disease (Corder et al., 1993).

### Comparison of three IQ populations.

Although there was no convincing pre-existing evidence of a correlation between IQ and polymorphic variation of CGG repeat numbers within the normal range (less than 52 repeats), we decided to analyse our samples received for fragile X testing and to compare this low IQ population (lower 3% of the intelligence distribution) with a high IQ population (members of British Mensa Ltd.). The requirement for membership of Mensa is an IQ of 132 or more (Wechsler scale) which places this group in the top 2% of the IQ distribution. The Mensa samples were obtained by requesting a 10 ml mouthwash from members in the Edinburgh area (of which there are approximately 480). To ensure anonymity, only the sex and the Mensa number of each individual was requested and the IQ corresponding to each membership number was obtained from Mensa headquarters. A control population of presumed average IQ consisted of mouthwash samples which were received in the laboratory as part of the cystic fibrosis prenatal screening trial and which were tested anonymously (though the sex of each was noted). The ages of the control population and the Mensa population were not requested. Out of approximately 480 Mensa members who were asked to participate in this study, 339 people responded (>70%); this is a very high response level for a research project and is similar to take-up rates for prenatal screening for CF in Edinburgh (Brock, 1996). This eagerness to participate may be a reflection of the nature of the study and the cohort being studied. Figure 6 shows the approximate distribution of the three IQ populations studied.



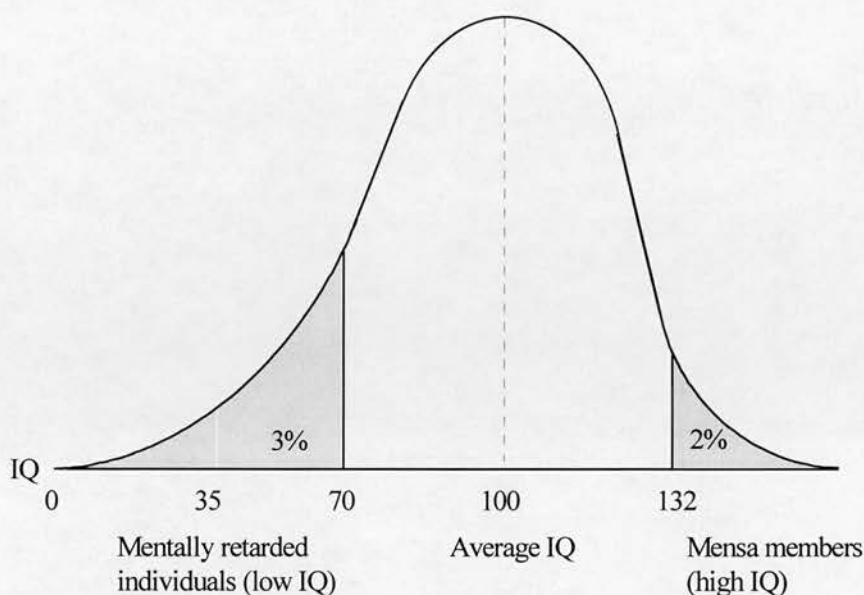


Figure 6. The three intellectually different populations which were analysed by PCR of the *FMRI* CGG repeat. (Not to scale).

A total of 1501 samples comprising leucocyte DNA from 781 mentally retarded patients, buccal cell DNA from 381 controls and buccal cell DNA from 339 Mensa members were analysed by PCR across the *FMRI* CGG repeat. PCRs were carried out using the radioactive method as described in 6.1.4.1 and PCR products analysed as in 6.1.4a. Results were obtained for 1377 of the samples (2.3.2). The size of the alleles in each case were compared to a female control known to consist of 20 and 30 repeats as compared to a size marker (*MspI* digested pBR322). An allele of 29 repeats was reported by Fu et al. (1991) to have an amplified length of 308 bp and an allele frequency histogram showed the mode to be 29 repeats. Later, Brown et al. (1993) determined by sequencing that the most common allele actually consists of 30 repeats. Although I did not sequence the CGG repeat in my control samples, I have assumed that the mode in the populations I have analysed is the same as the mode found by other groups, i.e. 30 repeats (the mode is universally 30 repeats although there are minor ethnic variations in allele distributions, see 2.3.2.2).



#### **2.2.4 Prenatal diagnosis of fragile X syndrome from 1991-1995**

During the five year period when I was collecting data on fragile X cases, a total of nine prenatal diagnoses were carried out in the laboratory. Our first prenatal diagnosis was carried out very soon after *FMRI* had been identified, in fact, the chorionic villus sampling was delayed so that the DNA probes (Ox1.9 and StB12.3) could be tested on the affected proband and other control samples (known affected male and female fragile X patients). This prenatal diagnosis (a twin pregnancy in which both male fetuses were predicted to be affected) was interesting in other respects and is described in detail in **2.3.3.1**. Later, other reports appeared in the literature from laboratories who had also carried out DNA based prenatal diagnoses (Hirst et al., 1991b; Dobkin et al., 1991). The fifth prenatal diagnosis carried out in our laboratory occurred in a pregnancy in which the fetus was predicted to be a normal transmitting male. Fetal blood sampling was necessary to guarantee that the premutation predicted in the CVS was truly a premutation (and not a compound premutation/full mutation) in the fetus itself (Strain et al., 1994a). This pregnancy is described in detail in **2.3.3.3**. The eighth prenatal diagnosis, although straightforward and predicted a normal male fetus, showed that there had been a cross-over between *DXS548* and *FRAXA* in a previous meiosis. This is described in more detail in **2.3.3.2**. The other pregnancies were predicted to be normal (3 female and 3 male fetuses) and proceeded to term. The detailed study of these unusual prenatal diagnoses have contributed both to our practical experience and to our understanding of the nature and timing of repeat expansion.

## **2.3 RESULTS**

### **2.3.1 Fragile X testing for developmental delay or mental retardation**

After 4 years and 3 months a total of 781 blood samples had been received in the laboratory for fragile X testing (Figure 7). Of these, 58 were eliminated from testing, either because they were duplicate samples or because they failed to produce DNA. 22 DNA extractions failed because, a) the sample was too small (less than 1 ml) or b) too old (the sample had been received in the cytogenetics laboratory and not directly from the clinician). When this occurred a repeat sample was requested, although, out of 22 repeats requested, fresh blood samples were obtained from only 11 patients.

Following the exclusion of duplicates and DNA extraction failures, 723 DNA samples were subsequently analysed by Southern blotting and PCR (Figure 8). Typical autoradiographs of a Southern blot and PCR products obtained in fragile X testing are shown in Figures 9 and 10 (pages 73 and 74).

The majority of samples were received from the Lothian area (71%) with the Borders region contributing 17%, and Fife 11%. The remainder of the samples (~1%) were from other Scottish regions; these were received before fragile X testing was available in all the Scottish Consortium centres.

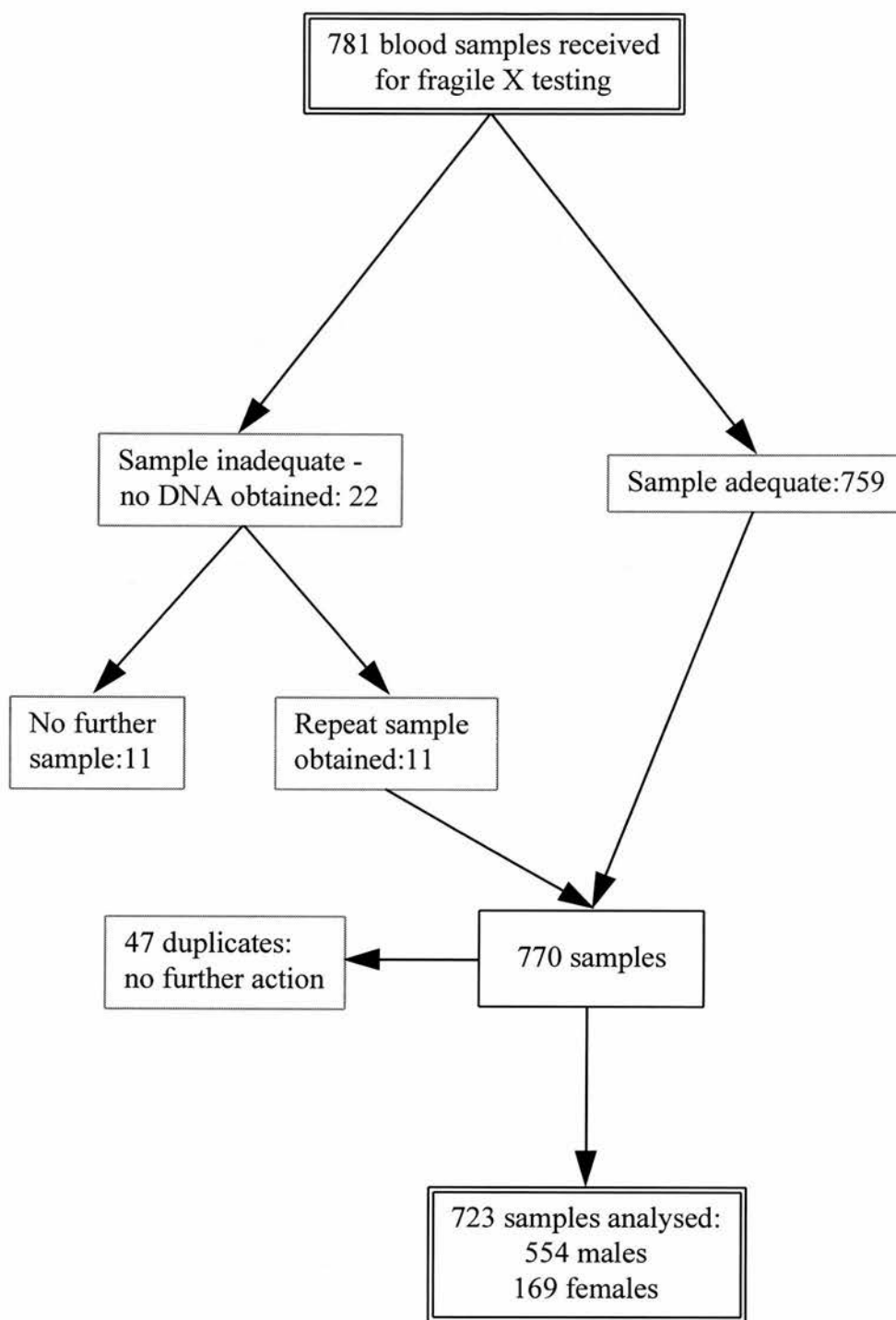


Figure 7. Synopsis of blood samples received for fragile X testing from 10/10/91 to 26/1/96.

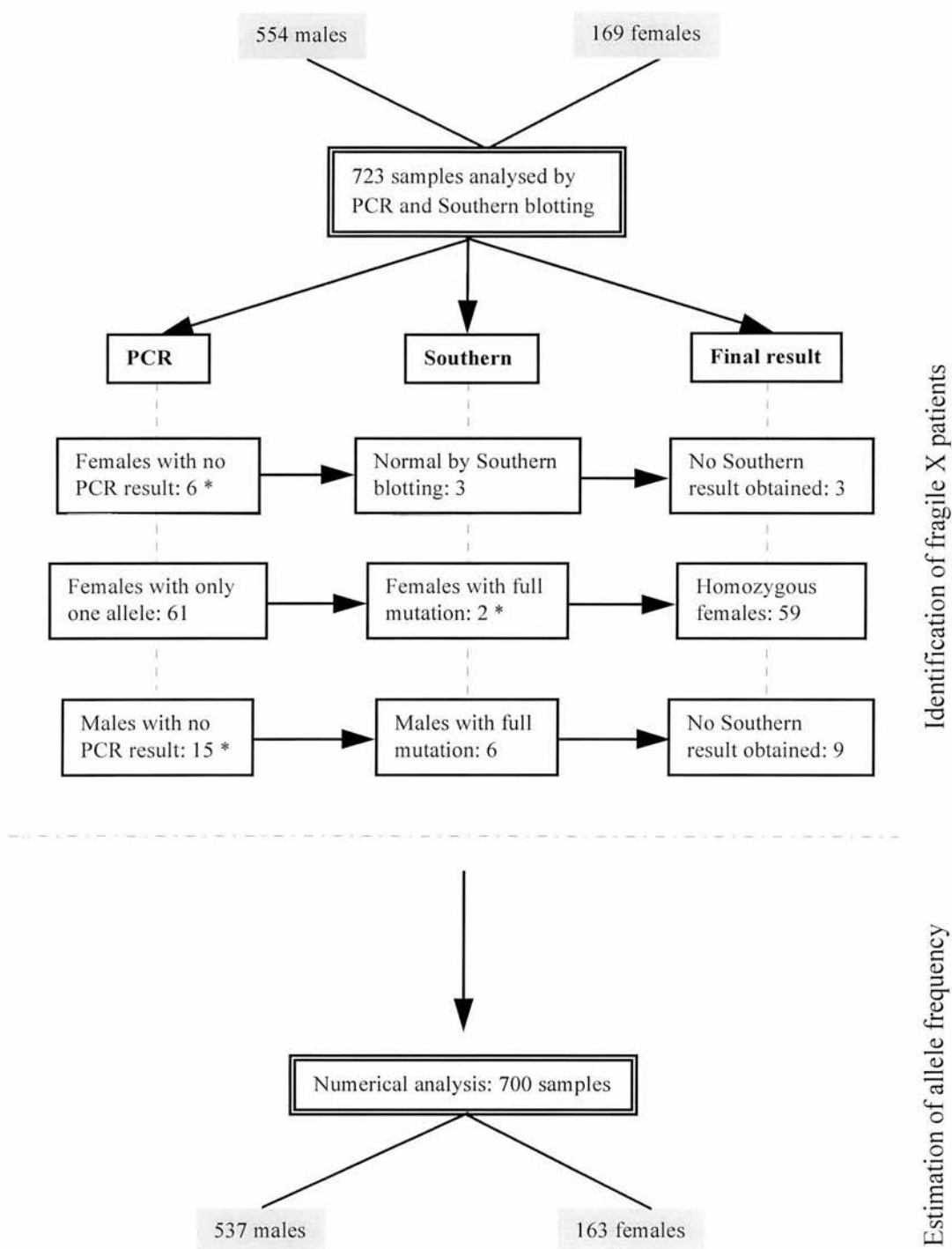


Figure 8. Summary of processing of DNA samples received for fragile X testing. The two females with a full mutation are sisters. After exclusion of samples marked \*, 700 DNA samples were left for numerical analysis of the CGG repeat.

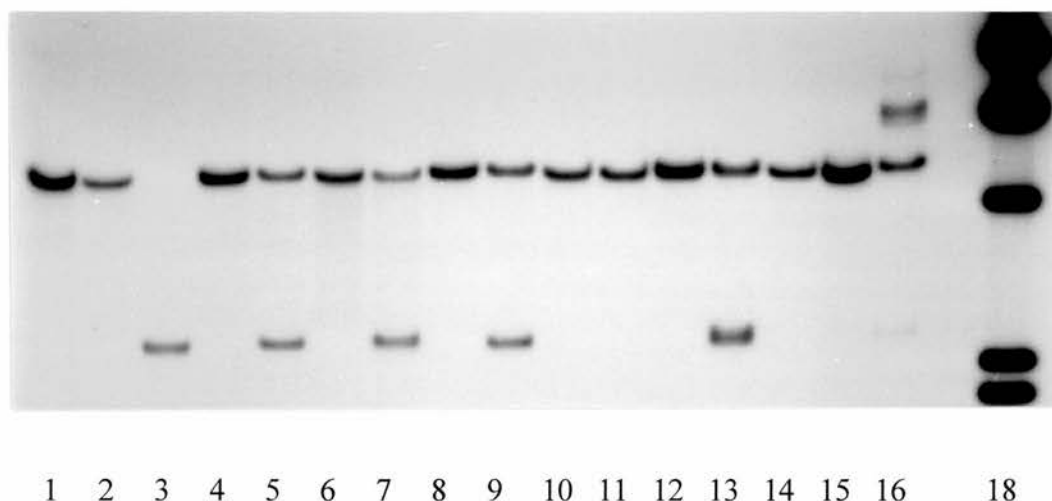


Figure 9. Typical autoradiograph of a Southern blot of DNA from ten mentally retarded individuals referred for fragile X testing. Lanes 1, 2, 4, 6, 8, 10, 11, 12, 14 and 15 contain DNA from males digested with *Hind*III. Lane 3 contains DNA from a male digested with *Hind*III and *Eag*I (the double digest was performed on this male sample because the sex of the patient was unknown at the time of the analysis, but was later confirmed to be male by PCR at the PAB locus). Lanes 5, 7, 9, and 13 contain DNA from females digested with *Hind*III and *Eag*I. Lane 16 contains DNA from a full mutation carrier female control digested with *Hind*III/*Eag*I. The marker in lane 17 is *Hind*III digested  $\lambda$  DNA; the bands shown here are 9kb, 6.6kb, 4.4kb, 2.2kb and 2.0kb. The bands at 2.5kb correspond to the unmethylated X chromosome in females or the male X (lane 3) and the bands at 5.0kb are either the X chromosome in males (*Hind*III digest) or the methylated X in females (*Hind*III/*Eag*I digest). The faintness of the band at 2.5kb in lane 16 may be due to either to incomplete *Eag*I digestion or to non-random X-inactivation (or a combination). This blot was hybridized with StB12.3.

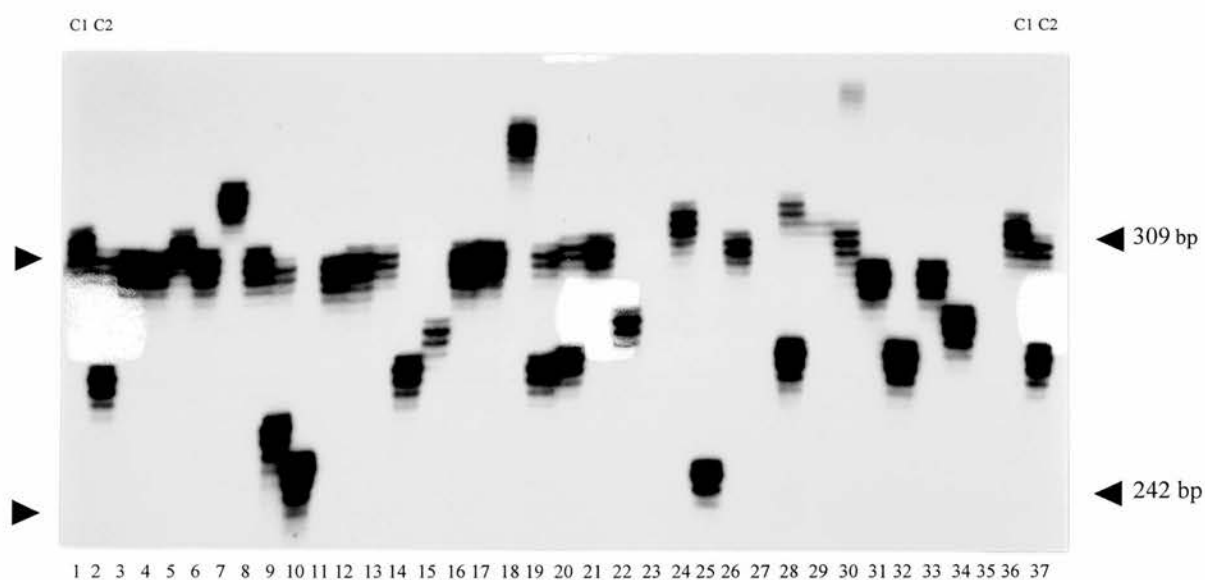


Figure 10. Typical autoradiograph of PCR products (amplified using primers **c** and **f**) showing the polymorphic nature of the *FMR1* CGG repeat. These are from a group of mentally retarded patients referred for fragile X testing. Lanes 1 and 36, and lanes 2 and 37 contain amplified DNA from a male control (C1) with a 32 repeat allele and a female control (C2) with 30 and 20 repeats, respectively. The arrows indicate the positions and sizes of the marker (*MspI* digested pBR322 DNA). The CGG repeat numbers in this group range from 13 repeats to 49 repeats. The three lanes in which there is no product consist of two PCR failures (lanes 23 and 27) and one male with a full mutation which was subsequently detected by Southern blotting (lane 35).



**2.3.1.1 Clinical presentation of patients referred for fragile X testing.**

For the 723 samples which were analysed by Southern blotting and PCR, clinical information about the patient was obtained from the form accompanying the blood sample, although for approximately 20% of samples, no information was available. The ages of the patients referred for fragile X testing varied from only a few weeks to 78 years with a mode of 4 years (Figure 11).

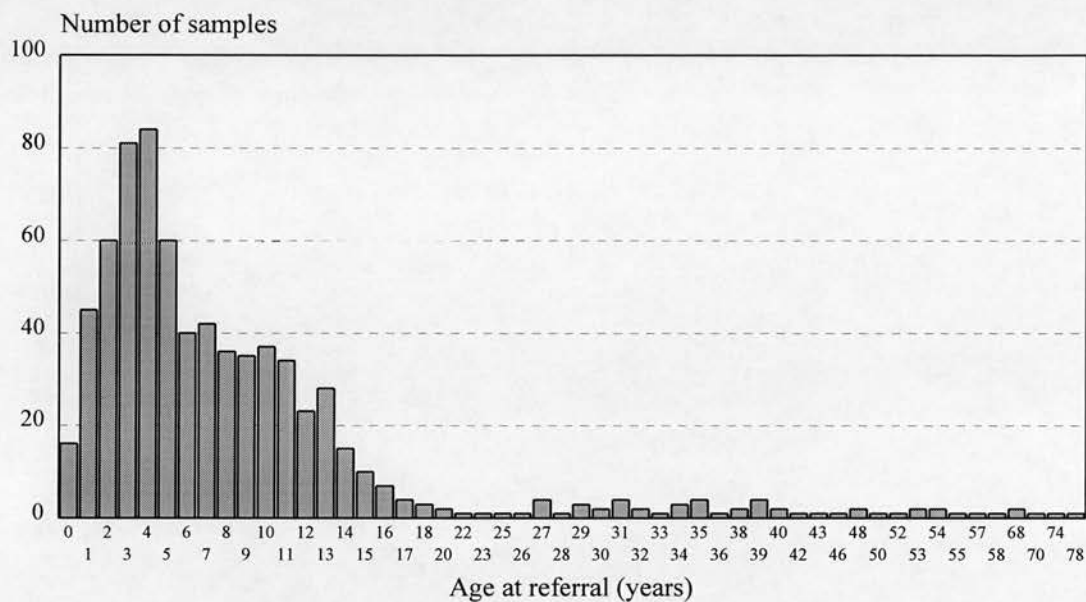


Figure 11. Histogram showing variation in age of patients referred for fragile X testing.

The initial strategy was to identify children with fragile X syndrome presenting as developmental delay in the pre-school years (5½ years or under); these made up 49.5% of the total referrals. However, early in the study, it was apparent that fragile X testing was required for a large number and variety of patients in all age groups. The remainder, therefore, consisted of school age children with learning difficulties, adolescents with mental retardation, and a small proportion (3.6%) who were resident in mental institutions in Lothian and Fife. As expected, the single most common clinical feature in both age groups was developmental delay or mental retardation (Figure 12), with the next most common feature being specific speech problems in the pre-school children and behavioural disorders in the older age groups.

Approximately 9% of the younger group (32 of 358) were referred because of autistic features or Asperger syndrome, but only 3% of the older group were reported to be autistic. None of these patients had an *FMR1* expansion. None of the patients in the mental institutions were found to have an expanded CGG repeat.

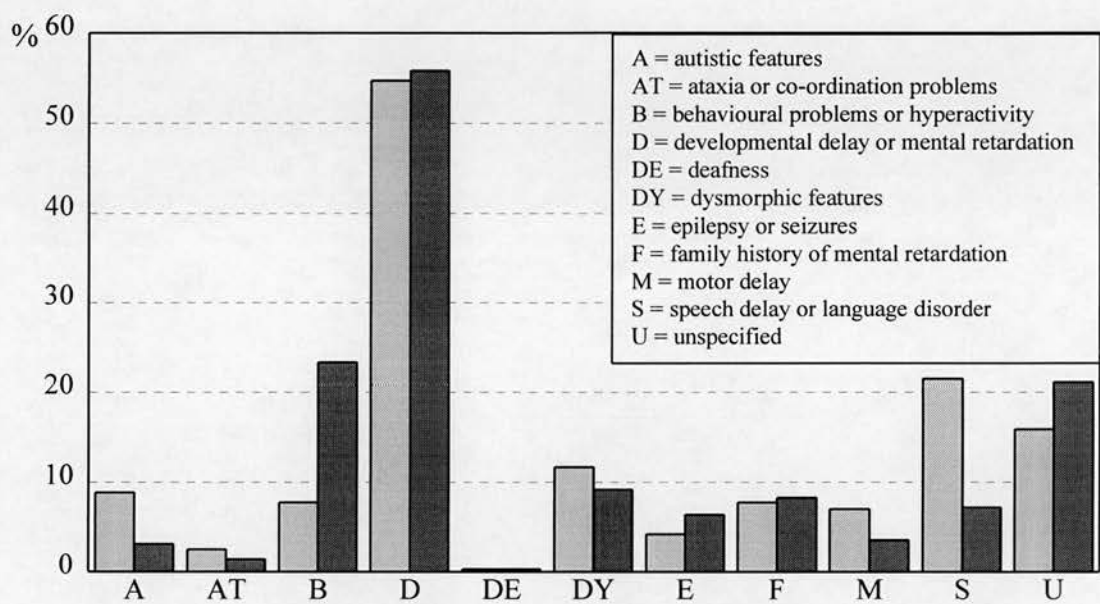


Figure 12. Histogram showing clinical features of patients referred for fragile X testing. Light shaded bars represent children aged 5½ years or younger; darker bars represent patients over 5½ years. As many of these features are not mutually exclusive, the sum of the figures is not 100%.

In constructing the histogram in Figure 12, I used all the information provided by the clinician who referred the sample. Obviously, some of the features listed above would not be considered diagnostic of fragile X syndrome if they occurred alone, for example, the two individuals who presented with deafness as part of the phenotype (one in each age group) had other features which may be relevant to fragile X syndrome; these were autism in one case and a family history of XLMR in the other. Likewise, a presenting feature of epilepsy or seizures would not be indicative of fragile X syndrome but may occur with other characteristics such as developmental delay.

### **2.3.1.2 Identification of eight new cases of fragile X syndrome**

This programme of testing developmentally delayed children in S.E. Scotland resulted in the identification of eight new cases (six males) of fragile X syndrome. The two females were sisters who had been shown to demonstrate the cytogenetic fragile site and were received for confirmation by molecular methods. Table 8 summarizes the clinical features recorded for each of these fragile X cases, and Table 9 shows the cytogenetic findings in each case. When a child was found to have a full mutation, a report was sent to the clinician on the basis of the results obtained from the initial sample; a repeat blood sample was not requested. For each positive identification, members of the immediate family of the proband were analysed, and in every case, the mother was a carrier of either a premutation or a full mutation (Figures 13 and 14, pages 82 and 83).

Of the eight new cases identified, the following observations were made about the full mutations seen on Southern blotting: cases 3 and 4 had condensed smears with the lower limit at approximately 9kb. Case 6 had a band which may have been at the upper end of the premutation range in addition to a full mutation smear above 6kb (the resolution of the gel did not allow accurate determination of the lower limit of the expansion). Methylation analysis, which would have differentiated between a large unmethylated premutation and a small methylated full mutation, was not carried out on this sample. Thus, it is unknown if this male is a methylation mosaic or a mosaic for small and large full mutations. The other cases had varying degrees of heterogeneity and smears above approximately 7kb, but were otherwise unremarkable. PCR analysis of each of the samples showed absence of product for each of the males and a single allele for the two females. There was no detectable signal in the premutation range for any of the samples.

Case no. (Family/individual)	Age	Sex	Clinical features
1 (Family A/III:1)	17 months	M	Motor and cognitive developmental delay. Large ears, coarse facies.
2 (Family B/III:2)	49 months	M	Delayed language development. Single palmar crease, mild clinodactyly (left hand). Large ears.
3 (Family C/III:2)	12 years	F	Specific learning difficulties especially with maths. Severe visual spatial problems. Long facies with slanting eyes.
4 (Family C/III:1)	14 years	F	Specific learning difficulties especially with maths. Relationship difficulties with peers and teachers. Long facies with slanting eyes.
5 (Family D/III:3)	23 months	M	Slow speech and slow in motor milestones. Mother's sister has two mentally retarded children. Prominent ears, triangular face.
6 (Family F/II:1)	35 months	M	Mild to moderate developmental delay. Large ears, thin face.
7 (Family E/IV:6)	27 months	M	Developmental delay.
8 (Family G/III:2)	22 months	M	Developmental delay

Table 8. Clinical features of the newly identified fragile X cases as detailed on the form accompanying the blood sample. Cases 3 and 4 are sisters who were first diagnosed by demonstration of the cytogenetic fragile site and were received in the laboratory for confirmation of diagnosis. The family/individual references in the first column denote the position of each case in the pedigrees A-G in Figures 13 and 14.

Case no.(from Table 8)	Sex	% of Fra(X) positive cells
1	M	No record
2	M	30%
3	F	44%
4	F	60%
5	M	No record
6	M	0%
7	M	12%
8	M	1 cell was found which may be Fra(X) +ve. 2 other cells were found with aberrant banding/morphology at distal Xq in 72 cells scored.

Table 9. Cytogenetic findings of the eight cases detected in this study. 100 cells were assessed in each case, apart from case 8 which was not referred as possible fragile X and therefore cells were not cultured in low folate medium. All cytogenetic testing was carried out at the Lothian Area Cytogenetics Laboratory, Royal Hospital for Sick Children, Edinburgh.

Case 6 is a false negative because this patient was not identified as having the cytogenetic fragile site but was shown to have a full mutation by DNA testing. The cytogenetic and DNA results of the other cases are in accordance where those samples were sent to cytogenetics specifically for fragile X testing and were therefore treated as such i.e. cultures were set up in low folate medium.

Following diagnosis of fragile X syndrome in these eight children, further DNA analysis was carried out on other family members. In every case the mother was tested and in some cases, the grandparents were also analysed. Knowing which grandparent had transmitted the mutation meant that siblings of the spouse of that grandparent could be excluded from further testing. The eight children belong to seven families (A-G) which are illustrated in Figures 13 and 14 (pages 82 and 83).

In family D, the mother of the affected boy was reported to have two cousins who are mentally retarded, but we cannot confirm whether this is due to fragile X syndrome because blood samples were not received from these children or their mother.

Although individual IV:6 in Family E (Figure 14) was originally identified as having fragile X syndrome through the screening programme, we discovered a few years later that this boy is actually a nephew of the mother (III:1) in whom a prenatal diagnosis was carried out (described in 2.3.3.1). The sisters III:1 and III:3 in Family E had lost contact with each other. For this reason, the affected boy IV:6 was treated as a newly identified case.

#### How does our detection rate compare with other centres?

Out of the 723 samples which were analysed, 358 (49.5%) were below the age of 5½. The six males who were identified as having a full mutation (Table 8) were very young (average age 2½ years) when they were first diagnosed which indicates that they are quite severely retarded. The two girls however, are much less severely affected and are attending a normal school. Of the 358 pre-school children, 280 were boys. Thus, the overall frequency of fragile X syndrome in this cohort is 2.2%, 2.1% for males only, and 2.6% for females only. Since the two girls who were identified



are sisters, they should ideally be treated as one positive identification. This reduces the overall frequency to 1.9%, and the frequency in females to 1.3%. The figures obtained in this study are comparable with those obtained by Slaney et al. (1995) who found four males with a full *FMRI* mutation out of 154 children (2.6%) attending special schools in Oxfordshire. In an earlier DNA based screening survey of mentally retarded children carried in southern England, Jacobs et al. (1993) found four males out of 254 children tested, giving an overall frequency of 1.6%. Hence, the frequencies of fragile X cases amongst children presenting with developmental delay, in the U.K., as detected by molecular methods, appear to be in the region of 1-2%. However, if the seven new cases identified in this study are taken as a proportion of all samples referred for fragile X testing (723), this figure becomes 0.97%. Therefore, the figures vary depending upon which subgroup of the population is used as the denominator. In a recent informal poll carried out by the British Society of Human Genetics (BSHG), the number of new cases of fragile X syndrome detected by DNA testing was 0.5% in most centres in the U.K., but it was felt that this could be increased with more discriminatory referral of patients. In order to produce a more efficient system the BSHG is in the process of collecting clinical data on each patient referred. The plan is to correlate those clinical features which are most often associated with a positive test result and to develop a scoring system whereby testing will only be carried out on patients with an appropriate score.

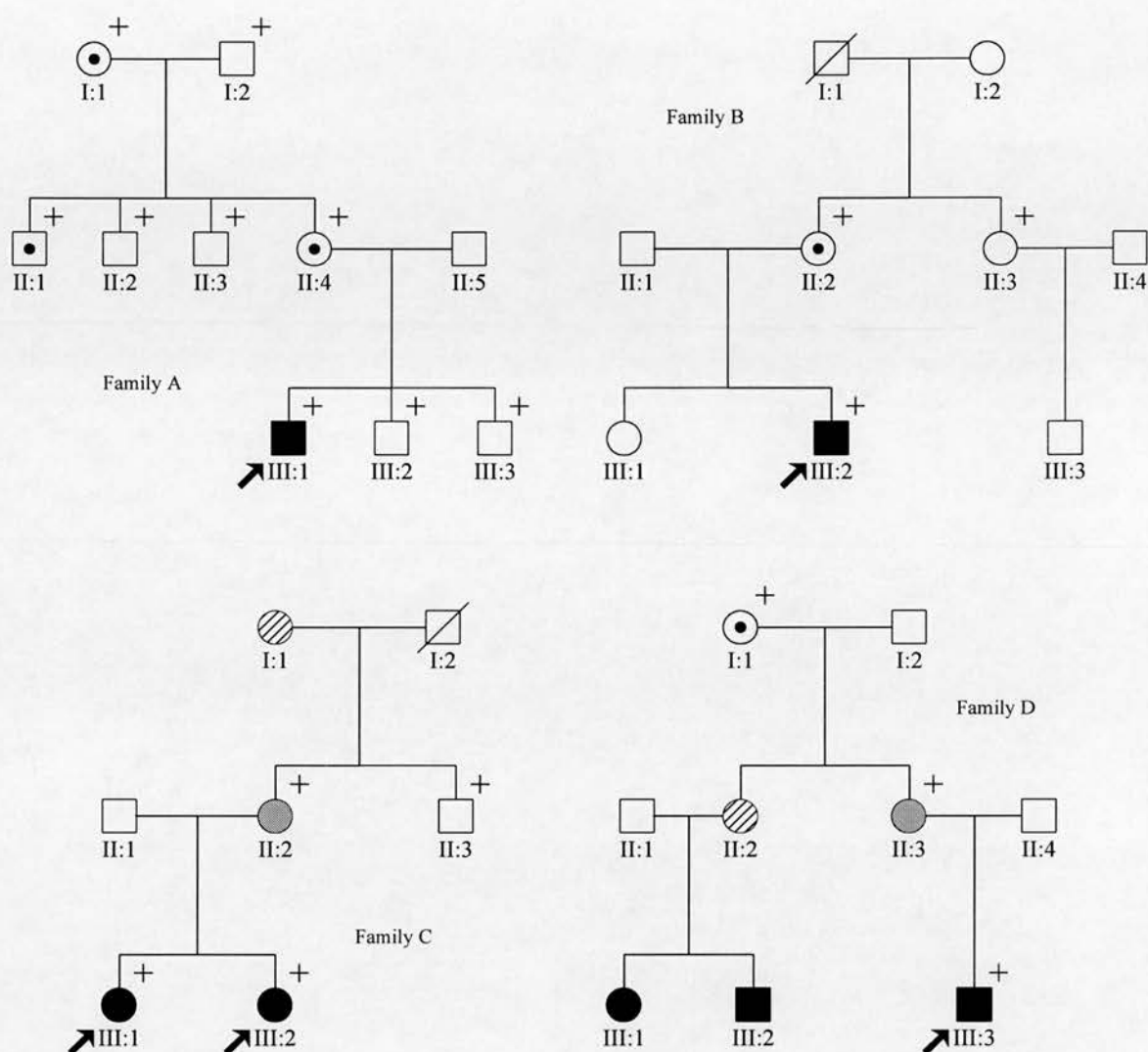


Figure 13. Pedigrees of families A, B, C and D. Individuals whose status was determined by DNA testing are indicated with a cross. Premutation carriers are represented by symbols with a dot, affected individuals are shown as filled black symbols and full mutation female carriers are represented by filled grey symbols. The individuals with a cross-hatched symbol are carriers of either a premutation or full mutation; they were not tested and their status cannot be inferred from the pedigree structure. The proband in each family (indicated by an arrow) was a mentally retarded individual who was first identified through DNA testing for fragile X syndrome.

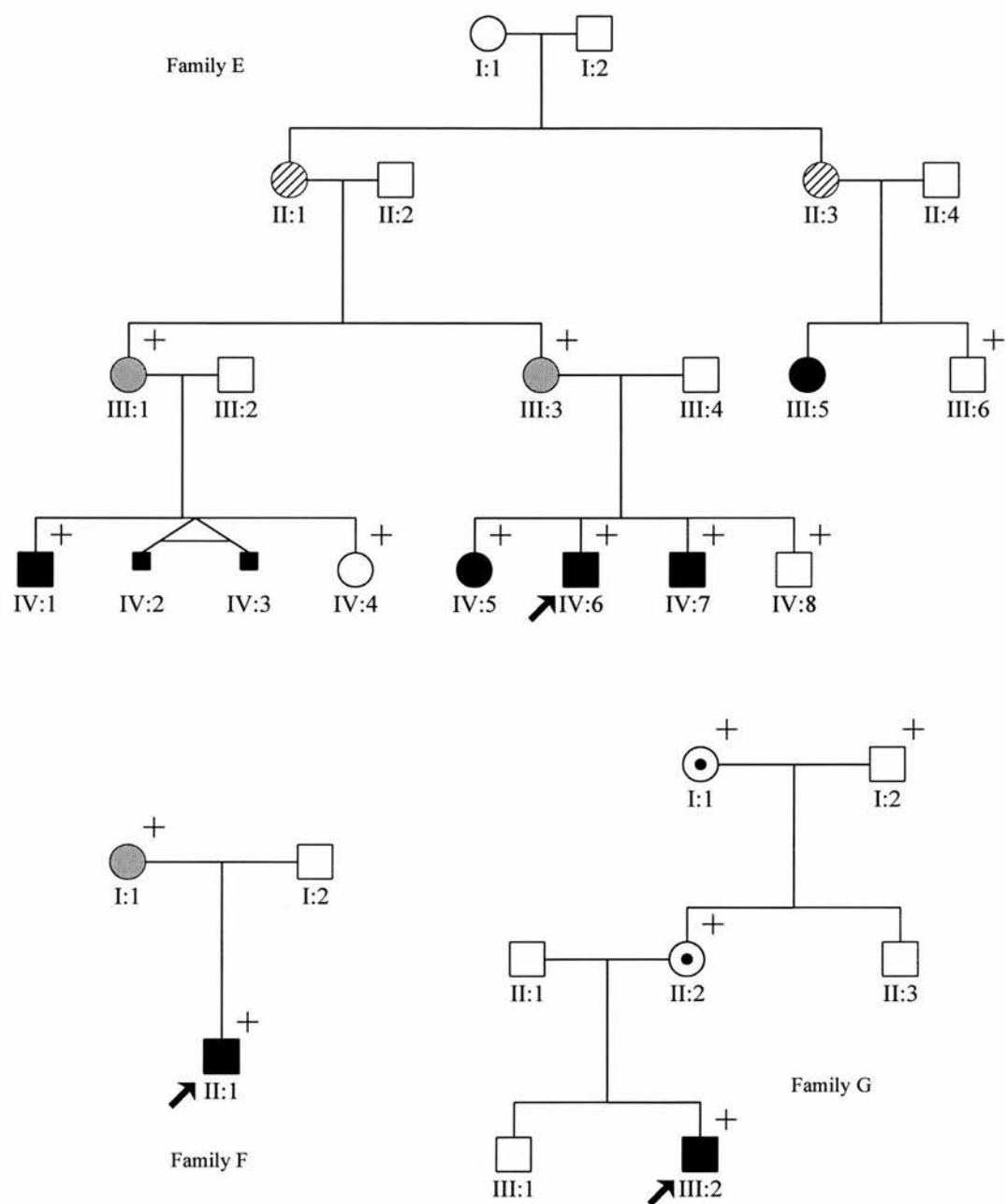


Figure 14. Pedigrees of families E, F and G. Symbol designations are as for Figure 13.

### **2.3.2 Analysis of the *FMRI* CGG repeat in three intellectually different populations**

In order to compare the allele frequencies of the *FMRI* CGG repeat in the low, average, and high IQ populations, a total of 1501 samples were analysed by PCR. These comprised 781 samples from mentally retarded individuals, 381 from normal controls (from the CF screening trial) and 339 from Mensa members. Results were obtained for 1377 of the 1501 samples and represented a total of 1741 alleles from 1014 males and 363 females. Table 10 summarizes these data.

### **2.3.3 Comparisons of CGG repeat distributions**

The allele distributions for each IQ subgroup of the population are illustrated in Figure 15. As shown in these histograms, the most common allele in each group consists of 30 repeats with two other major peaks at 20 and 23 repeats, and a minor peak at 38-40 repeats. These allele frequency distributions are similar to those obtained by other groups (Fu et al., 1991; Eichler et al., 1995). Eichler et al. (1995) however, reported that the frequency distribution was trimodal with peaks at 20, 30 and 40 repeats, yet it is clear from their data that the distribution in the 406 normal X chromosomes they analysed, is similar to that obtained in this study i.e. peaks at 20, 23, and 30 repeats. Arinami et al. (1993) analysed the CGG repeat in 824 X chromosomes in a Japanese population and found the distribution to be slightly different; the mode was 29 repeats, the next most common allele consisted of 36 repeats and a smaller peak was present at 23 repeats. Eichler et al. (1995) found that although there is considerable variability in the distribution of the interspersed AGG repeats among different ethnic groups, the mode is universally 29 or 30 repeats.

In the CGG repeat analysis study described in this chapter, two alleles were found in two DNA samples which were in the borderline normal/premutation range, one in the low IQ group and one in the high IQ group. The presence of this large allele in the low IQ group is probably not related to the mental retardation in this patient who was an eleven year old girl with alleles estimated to consist of 30 and 55 repeats. The Mensa member was a female with alleles consisting of 40 and 56 repeats.

However, as none of the CGG repeats were sequenced, we do not know whether these large alleles were interspersed with AGG repeats and therefore at higher risk of becoming premutations in subsequent generations.

	Mentally retarded patients (low IQ)	Controls (average IQ)	Mensa members (high IQ)
Number of samples received	781	381	339
Type of sample	blood	blood/MW	MW
Number of results obtained after exclusion for DNA/PCR failure, duplicates etc.	700	353	324
PCR failure rate (%)	2.1	7.3	4.4
Male to female ratio	537:163	292:61	185:139
Total number of alleles <sup>a</sup>	864	414	463
Percentage of heterozygous females <sup>b</sup>	63.8 (74.2)*	63.9	61.9
Predicted heterozygosity <sup>c</sup>	81.8 (81.6)*	79.8	82.0
Predicted heterozygosity using male data only <sup>d</sup>	82.0	81.4	83.7
Predicted heterozygosity using female data only <sup>e</sup>	81.4	74.8	81.1

Table 10. Comparison of data on the *FMRI* CGG repeat in three intellectually different populations. <sup>a</sup>The mentally retarded population included one 46,XX male. The observed heterozygosity<sup>b</sup> was calculated for alleles which differed by 1 or more repeats. The predicted heterozygosity<sup>c</sup> was calculated from the allele frequencies (Table 12) using the Hardy-Weinberg equation. Since initial results suggested a discrepancy between observed and expected heterozygosities, the predicted heterozygosity<sup>d</sup> was calculated using data from males only to obtain the “true” heterozygosity rate. Figures using female data only<sup>e</sup> are given for comparison. MW=mouthwash sample. The figures in brackets marked\* are the heterozygosities calculated after re-analysis of the data (see text).

The allele frequencies for each population are shown as histograms in Figure 15 and in table form in Table 11 (page 87). The frequencies of the three populations combined are shown as a histogram in Figure 16 (page 88).

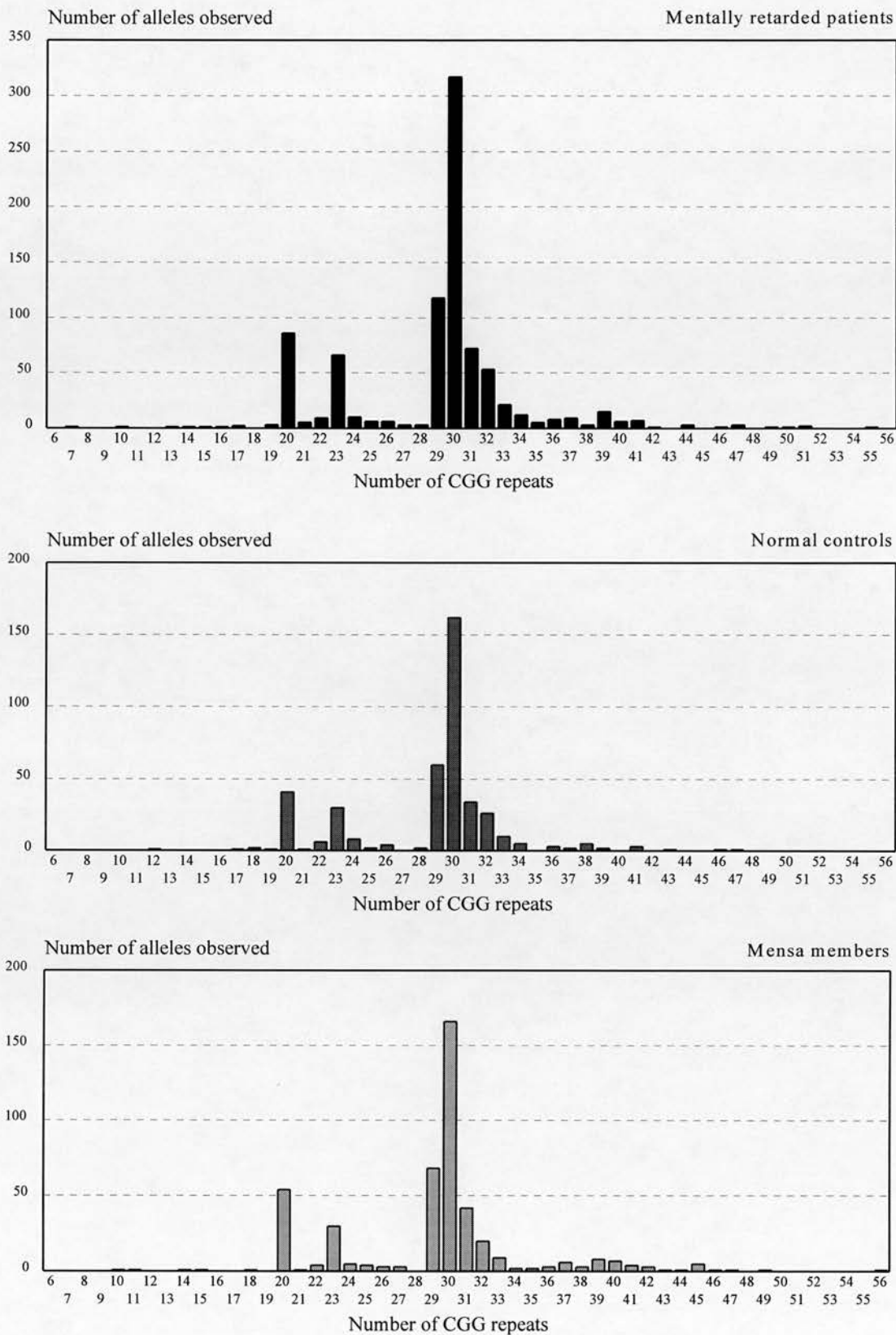


Figure 15. Histograms showing the allele distributions of the *FMRI* CGG repeat in the three different IQ populations studied. These were constructed from the data in Table 11.



	Mentally retarded individuals		Controls		Mensa	
Number of repeats	Number of alleles	% of total	Number of alleles	% of total	Number of alleles	% of total
7	1	0.12	0	0.00	0	0.00
10	1	0.12	0	0.00	1	0.22
11	0	0.00	0	0.00	1	0.22
12	0	0.00	1	0.24	0	0.00
13	1	0.12	0	0.00	0	0.00
14	1	0.12	0	0.00	1	0.22
15	1	0.12	0	0.00	1	0.22
16	1	0.12	0	0.00	0	0.00
17	2	0.23	1	0.24	0	0.00
18	0	0.00	2	0.48	1	0.22
19	3	0.35	1	0.24	0	0.00
20	86	9.95	41	9.90	54	11.66
21	5	0.58	1	0.24	1	0.22
22	9	1.04	6	1.45	4	0.86
23	66	7.64	30	7.25	30	6.48
24	10	1.16	8	1.93	5	1.08
25	6	0.69	2	0.48	4	0.86
26	6	0.69	4	0.97	3	0.65
27	3	0.35	0	0.00	3	0.65
28	3	0.35	2	0.48	0	0.00
29	118	13.66	60	14.49	68	14.69
30	317	36.69	162	39.13	166	35.85
31	72	8.33	34	8.21	42	9.07
32	53	6.13	26	6.28	20	4.32
33	21	2.43	10	2.42	9	1.94
34	12	1.39	5	1.21	2	0.43
35	5	0.58	0	0.00	2	0.43
36	8	0.93	3	0.72	3	0.65
37	9	1.04	2	0.48	6	1.30
38	3	0.35	5	1.21	3	0.65
39	15	1.74	2	0.48	8	1.73
40	6	0.69	0	0.00	7	1.51
41	7	0.81	3	0.72	4	0.86
42	1	0.12	0	0.00	3	0.65
43	0	0.00	1	0.24	1	0.22
44	3	0.35	0	0.00	1	0.22
45	0	0.00	0	0.00	5	1.08
46	1	0.12	1	0.24	1	0.22
47	3	0.35	1	0.24	1	0.22
49	1	0.12	0	0.00	1	0.22
50	1	0.12	0	0.00	0	0.00
51	2	0.23	0	0.00	0	0.00
55	1	0.12	0	0.00	0	0.00
56	0	0.00	0	0.00	1	0.22
<b>Σ</b>	<b>864</b>		<b>414</b>		<b>463</b>	

Table 11. Numbers of CGG alleles and their frequencies as a percentage of the total.

Alleles consisting of 6, 8, 9, 48, 52, 53, and 54 repeats were not represented in this study.

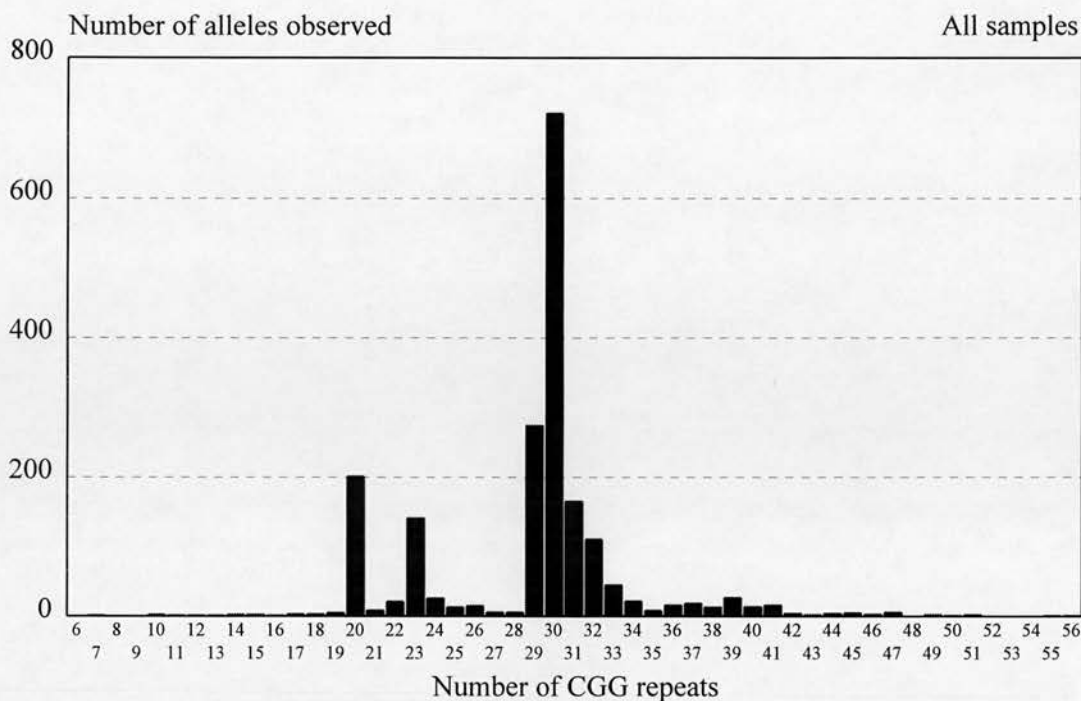


Figure 16. Histogram showing combined frequencies of all three IQ populations.

### 2.3.3.1 Calculation of heterozygote frequencies

Using the repeat number data obtained for each of the three IQ population subgroups, as tabulated in Table 12, the following were calculated: firstly, the number of females in each cohort with two alleles which differed by one or more repeats (observed heterozygosity), and second, the predicted heterozygote frequencies for each set using the Hardy-Weinberg equation. It was clear from these results (Table 10, page 85) that there was a large discrepancy between the observed and predicted heterozygote frequencies (an average of 18% for the three groups), so the predicted heterozygosities were then calculated using data from the males only in each group. Finally, for comparison, the frequencies were calculated using data from the females only.

#### Why is there a discrepancy?

The observed heterozygosity in the low IQ group was 63.8%, i.e. 104 females were heterozygous. The number of females expected to have two distinguishable alleles,

according to the Hardy-Weinberg equation is 134. The most likely explanation for the discrepancy is that females who were heterozygous for two closely spaced alleles were actually scored as homozygotes. Other explanations are either that the population has not yet reached Hardy-Weinberg equilibrium, or that there is a preponderance of a particular ethnic group among the three populations analysed. Both of these latter alternatives are extremely unlikely. Although Fu et al. (1991) found no significant differences between Caucasian, Black, Hispanic and Asian races in the 492 X chromosomes they analysed by PCR, more recent data (Arinami et al., 1993; Eichler et al., 1995) shows that while the overall distributions of CGG repeats among different races are similar, there are ethnic differences in the percentage heterozygosities. For example, African-Americans and Caucasians show the greatest variation with 87.3% and 88% (Eichler et al., 1995) while the Hispanic and Asian groups show predicted heterozygosities of 73.1% and 67.5% respectively. The high level of heterozygosity in the Caucasian group is due to the prevalence of the 20 CGG repeat allele (10.A.9) which is rare or absent in other populations. Other causes of deviation from Hardy-Weinberg equilibrium, related to ethnic differences, could be caused by consanguineous marriages which would result in an increase in the number of homozygotes. However, the majority of individuals in the three populations analysed here are likely to be of Caucasian descent, although it is probable that approximately 2% of the low IQ cohort may be of non-Caucasian extraction judging by the surname of the patient. Even if the surname of a child could be used as an indicator of race, the small number involved in this cohort is unlikely to have any effect on the heterozygote frequencies.

#### The differences may be a PCR related phenomenon

The discrepancies between observed and predicted heterozygosities were evident in all three subgroups and were presumed to be due to incorrect scoring of the alleles on the autoradiograph. In order to determine whether the problem was specific to the method I was using, I re-analysed the females (low IQ cohort only) who had been scored as homozygous with the radioactive method, by using a fluorescent PCR method (6.1.5). The rationale for this was that as only one of the primers is labelled

and therefore only one DNA strand of the PCR product will be fluorescent this should theoretically make scoring of closely spaced alleles easier. Figure 17 (page 92) shows examples of A.L.F. traces of *FMRI* CGG PCR products from females who were originally scored as homozygous by the radioactive method. When the homozygous females were re-analysed by fluorescent PCR in this way, seventeen females were subsequently additionally scored as heterozygous. This increased the observed heterozygosity to 74.2% and reduced the difference between observed and predicted heterozygosities in this group to 7%. The seventeen “new” heterozygotes had little effect on the total predicted heterozygosity (81.6% compared to 81.8% previously). This re-analysis was only carried out on the low IQ group.

#### Implications for fragile X testing using PCR alone

If PCR was the only method being used to determine the presence of an *FMRI* CGG repeat expansion, it would be necessary to exclude normal females by the detection of two distinguishable normal alleles. The number of shadow bands seen on denaturing polyacrylamide gels may be related to the nature of the CGG repeat with pure repeats (no AGG interruptions) giving a wider spread (Snow et al., 1994). It is possible, however, that in a female this could be misinterpreted as two alleles which differed by only one repeat. For this reason, the females in the low IQ group were further classified according to differences in size between alleles: Table 12 compares the results obtained with the radioactive PCR and the corrected results after re-analysis of the homozygous females by fluorescent PCR.

Number of females with:	Radioactive PCR	Fluorescent PCR
Alleles of the same repeat number	59	42
Alleles which differ by 1 repeat	14	30
Alleles which differ by 2 repeats	8	8
Alleles which differ by >2 repeats	82*	83*
<b>Total</b>	<b>163</b>	<b>163</b>

Table 12. Comparison of results obtained using radioactive PCR (internal labelling with  $\alpha$ -<sup>32</sup>P-dCTP) and a fluorescent PCR (one primer end labelled with fluorescein).

### Which PCR method is more discriminatory?

As shown in Table 12, the number of females with alleles differing by 2 or more repeats is 55% and 56% for the radioactive and fluorescent methods respectively, and therefore if this criterion was used in any form of screening for fragile X heterozygotes (for example, in an antenatal setting), it would imply that the remaining 45% of female samples would have to proceed to expensive, labour intensive and lengthy Southern blotting. The fluorescent PCR appears to be better at distinguishing closely spaced alleles (3bp different) but allele differences greater than this are detected equally by both labelling methods. The female DNA sample (marked with a \* in Table 12) which moved from the homozygous category to the >2 repeat category was an example of preferential amplification but was unusual in that the larger allele was hardly noticeable on the autoradiograph, yet quite easily discernible on the A.L.F. trace. This sample was recorded as 22, 22 by the radioactive method but as 22, 38 by the fluorescent method.

### Other methods of detection

Only two different methods of detection of PCR products have been presented here. Various laboratories have had success with other non-radioactive detection methods such as silver staining of PCR products in denaturing polyacrylamide gels, ethidium bromide staining of products in agarose gels (Wang et al., 1995), and a chemiluminescent detection method using blotted PCR products and an alkaline phosphatase labelled (CGG)<sub>5</sub> probe (Brown et al., 1993). However, all methods have one thing in common; they rely on a good yield of PCR product which is dependent upon the length of the repeat. I think the most promising PCR method (which does not use primers flanking the CGG repeat) is the MVR-PCR related technique which requires that primers anneal at every triplet within the repeat and therefore the number of CGGs in an expanded repeat is irrelevant.



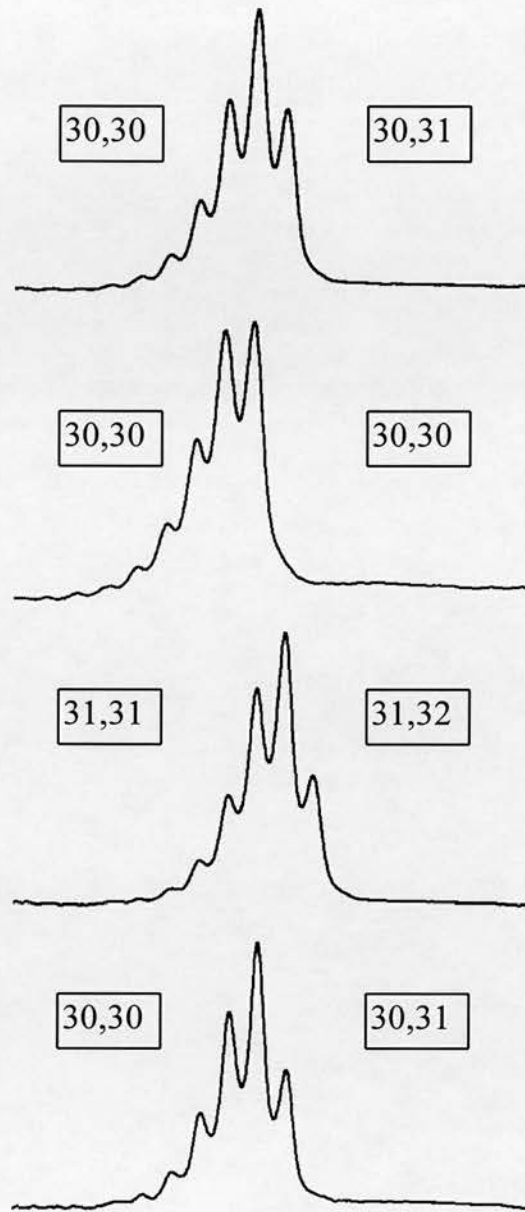


Figure 17. Examples of A.L.F. traces of the *FMRI* CGG PCR in DNA from females who were originally scored as homozygous by the radioactive PCR method. Numbers on the left of the traces are the numbers of repeats estimated to be in each allele using the radioactive PCR, numbers on the right are the corrected numbers from the fluorescent PCR results. The sample which was homozygous by both methods was analysed by PCR using a  $\gamma$ - $^{32}\text{P}$ -ATP labelled primer.



2.3.3.2 Are *FMRI* CGG repeat number and IQ correlated?

For this part of the study, 339 mouthwash samples were received from Mensa members in the Edinburgh area. DNA was extracted from each one and PCR across the *FMRI* CGG repeat was carried out as in 6.1.4.1 using the primers c and f. Results were obtained from 324 of the samples; if a PCR failed it was repeated only once more. The allele distribution is shown in Figure 15 (page 86, bottom graph) and appears to be the same basic shape as the distribution derived from DNA from people with average IQs (Figure 15, middle graph). Figure 18 illustrates the range of IQs in the Mensa members where this information was available.

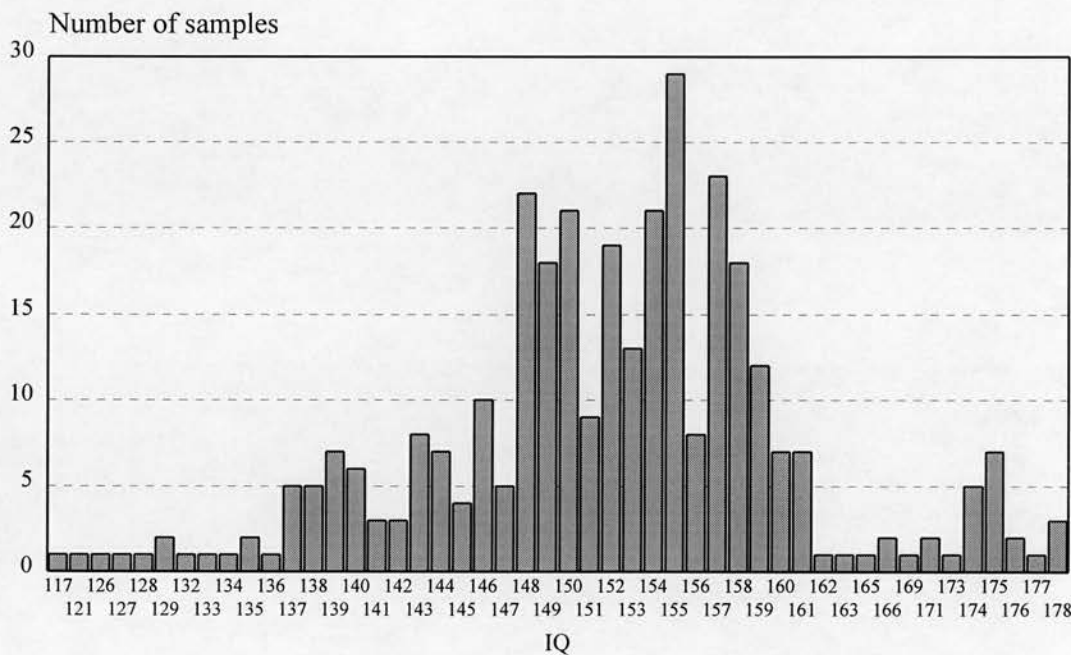


Figure 18. Histogram showing the distribution of IQ in the Mensa samples (where IQ is known). These scores were obtained using the Cattell iii B Verbal IQ test (information provided by British Mensa Ltd.).

In order to assess whether there is a correlation between an individual’s IQ and CGG repeat number, a scatter diagram was constructed using the Mensa data only and is shown in Figure 19. A scatter diagram could not be constructed for the mentally

retarded individuals or the controls because the absolute IQ values of these individuals were not known.

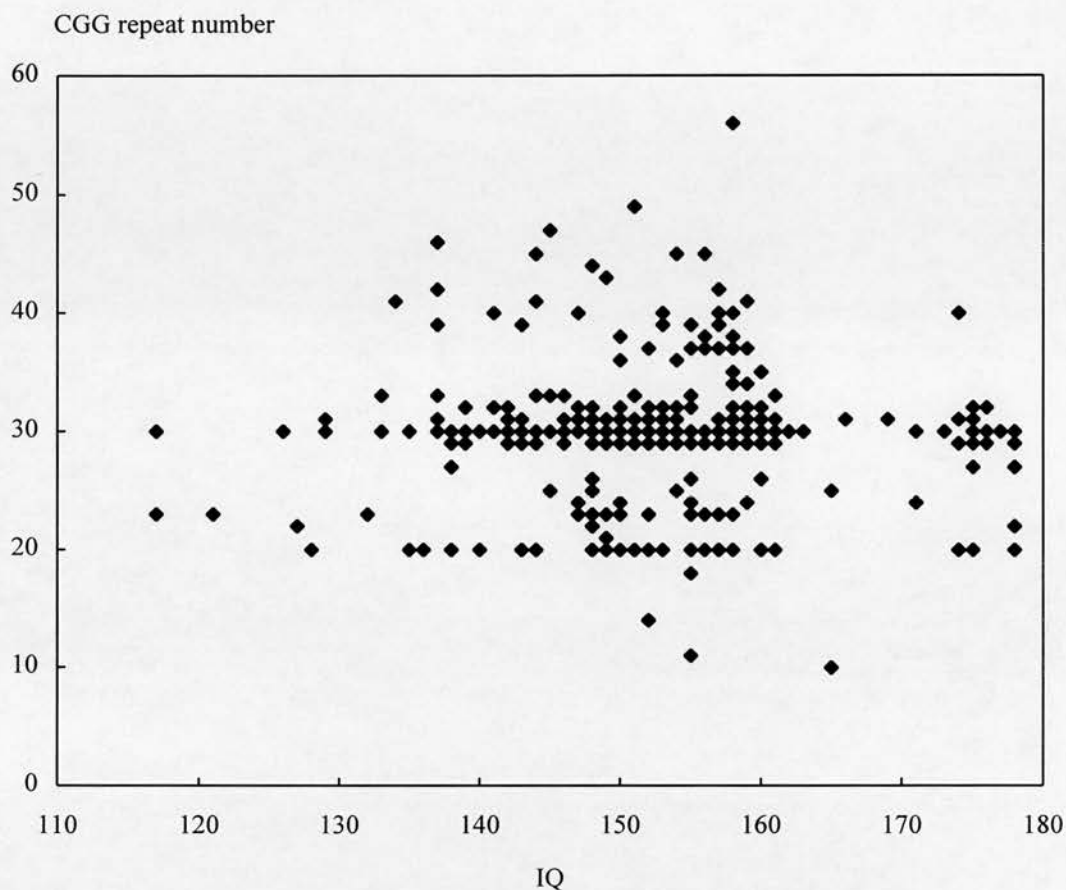


Figure 19. Scatter diagram showing the relationship between IQ in the Mensa members and *FMRI* CGG repeat number. Some individuals are not represented on this diagram, either because a PCR result was not obtained or because the IQ score of that individual was not available. 451 alleles out of a total of 463 are represented in this diagram.

#### No correlation between IQ and CGG repeat number

As shown in Figure 19, there is no correlation between IQ and number of CGG repeats (correlation coefficient = 0.018724). In view of this, there seemed to be little point in pursuing this investigation. Since the allele frequencies of each cohort are almost identical, the three populations can be combined and used as a “control” population for other studies.

### 2.3.3 Prenatal diagnosis of fragile X syndrome

In the five year period since the identification of *FMR1*, a total of nine prenatal diagnoses have been carried out in this laboratory. As described previously (2.2.4), six of these posed no technical or clinical problems; normal fetuses were predicted and the pregnancies proceeded to term. Two of the remaining pregnancies did not cause any technical difficulties as such, but interesting observations were made in both cases. The third was of special interest because it occurred at a time when new evidence was emerging about the timing of *FMR1* CGG repeat expansion. The first prenatal diagnosis occurred in a family in which a woman presented in her second pregnancy, a short time after the *FMR1* gene had been identified.

#### 2.3.3.1 Prenatal diagnosis of affected fetuses in a twin pregnancy.

##### Case report

The pedigree of Family E is shown in Figure 20.

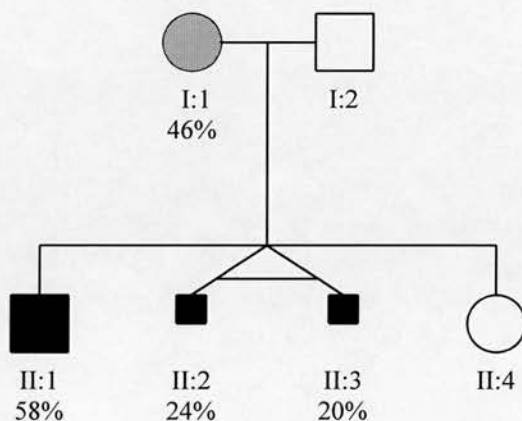


Figure 20. Pedigree of family E. The female carrier of a full mutation is shaded grey, affected males are shown as filled black symbols. The percentage of cells shown to have the cyto-genetic fragile site are shown below the symbols.

The proband in this family had been demonstrated to have the cytogenetic fragile site in 58% of cells and his mother (I:1) who had phenotypic features of fragile X syndrome demonstrated the site in 46% of her cells. Early in her second pregnancy, the mother was found to be carrying twins and ultrasound scanning indicated that the fetuses were in separate amniotic sacs. CV biopsies were taken (one trans-abdominally and the other trans-cervically) from each placenta at 11½ weeks and DNA analyses were carried out as described previously (2.1.3.19 and 6.1). The fetuses were shown to be male by PCR at the PAB locus (Ellis et al., 1990) and also demonstrated to be monozygotic twins by multilocus fingerprinting using the probe 33.15 (Jeffreys et al., 1985 and see 6.1.6). Southern blot analysis using StB12.3 demonstrated that both fetuses had a full mutation of approximately 6kb (Figure 21, lanes 3 and 4), which appeared slightly smaller and much less heterogeneous than the full mutation in both the proband and the mother.

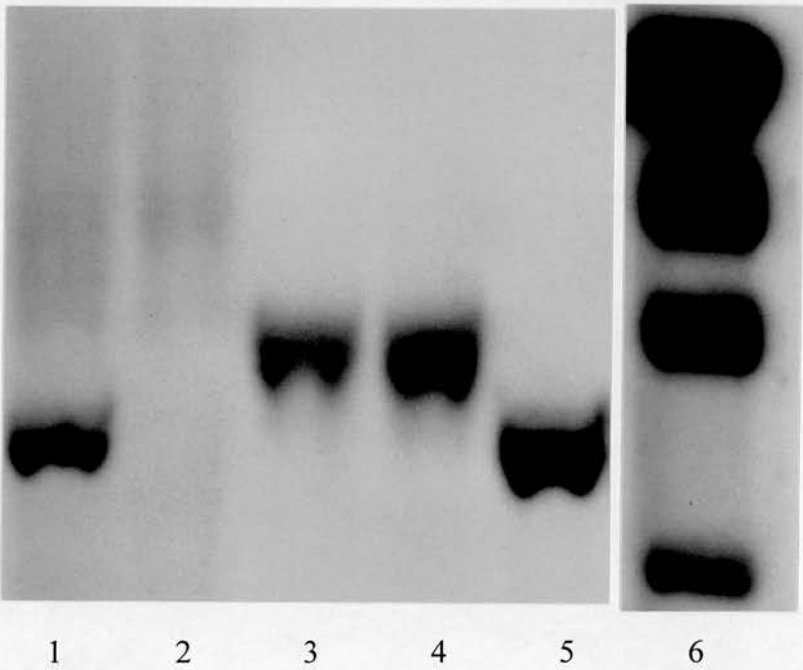


Figure 21. DNA analysis of family E by Southern blotting on *Eco*RI digested DNA. Lane 1 (mother), lane 2 (affected proband), lanes 3 and 4 (CVS DNA from twin fetuses), lane 5 (normal male). The marker (lane 6) is *Hind*III digested  $\lambda$  DNA; the bands shown here are 23kb, 9kb, 6.6kb and 4.4kb. This blot was hybridized with StB12.3.

Analysis of the microsatellite repeat, *DXS548*, also confirmed that both fetuses had inherited the same haplotype as the affected boy from their heterozygous mother.

On the basis of these collective data, the pregnancy was terminated at 18 weeks gestation. For each aborted fetus, DNA was extracted from heart, skull, cord, placenta, skeletal muscle, and gut. From one or other fetus, DNA was also extracted from brain, skin, testis, eye, tongue, adrenal, kidney, and cartilage. Southern blotting showed that the size and appearance of the band was identical in all tissues examined, including the CVS tissue (Figure 22). The full mutation in the mother appeared as a heterogeneous smear in the region of 6-8kb (Figure 22, lane 1); when compared to the CVS from one twin (lane 2) and fetal tissues from both twins (lanes 3 to 14), all showed discrete bands of approximately 6kb. The methylation status at the *FMRI* CpG island was not assessed in these cases.

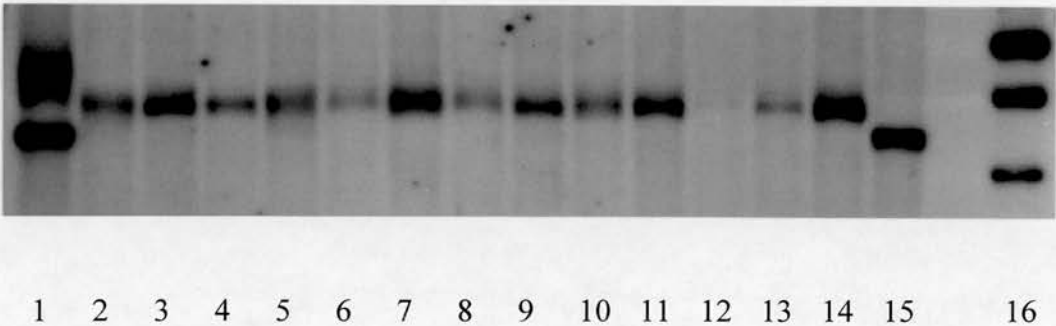


Figure 22. DNA analysis of CVS and fetal tissues from prenatal diagnosis of family E. Amounts of DNA vary between each lane because of the presence of RNA (samples were not RNase treated). DNAs were digested with *HindIII* and the blot was hybridised with StB12.3. Lane 1: full mutation carrier mother; lane 2: CVS DNA from one fetus; lanes 3-14: DNA from termination tissue from one or other twin (brain, heart, placenta, skull, eye, lung, gut, muscle, kidney, adrenal, tongue, testis); lane 15: normal male control; lane 16: Marker is *HindIII* digested  $\lambda$  DNA. Bands shown here are 9kb, 6.6kb and 4.4kb.



The appearance and size of the full mutation in the CV and fetal tissues compared to that in the mother suggests that the mutation transmitted by the mother, was substantially smaller (and perhaps a premutation) than the majority of alleles present in her blood. Another interesting observation from this case is that both twins have identical patterns, which suggests that the events leading to somatic mosaicism were completed before the time of division of the zygote into two embryos. It is unknown whether the twins were monochorionic or dichorionic although they were known to be diamniotic (monoamniotic twins result from a later form of twinning and often do not survive). In about 25% of cases, twinning occurs before the third day of development, but for the majority of monozygotic twins, twinning occurs between the third and eighth day, by which time the differentiation of the blastocyst has proceeded too far to allow duplication of the chorion. If expansion only occurs mitotically, then it must occur sometime after day 5 when the separation of the gametic and somatic cell lineages occur. Therefore, if the twins in family E are monochorionic then the expansion and subsequent stabilization of somatic mosaicism can be confined to between day 5 and 8, but if they are dichorionic, then the expansion may have occurred around day 5.

Nine months after the twin pregnancy was terminated, the mother (I:1) presented for prenatal diagnosis in her third pregnancy. A CV biopsy was taken and the fetus was shown to be female by the PAB PCR (Ellis et al., 1990). DNA analysis by Southern blotting and PCR revealed that the fetus had inherited the mother's normal X chromosome and the presence of a paternal X was shown by PCR across the CGG repeat. The pregnancy continued to term.

#### **2.3.3.2 Recombination between *DXS548* and *FRAXA* in a prenatal diagnosis**

This prenatal diagnosis occurred in a family in which the proband was identified through the programme of fragile X testing for mental retardation. He was also the first positive identification as a result of DNA testing for fragile X in this laboratory. The pedigree is shown in Figure 13 (page 82, Family A). Following the identification of the affected male in this family, other family members were tested,



including the mother, both grandparents, and three uncles of the affected boy. All individuals were tested by Southern blotting, PCR across the *FMRI* CGG repeat, and by PCR of the microsatellite *DXS548*. The *DXS548* and *FMRI* CGG results are shown in Figure 23.

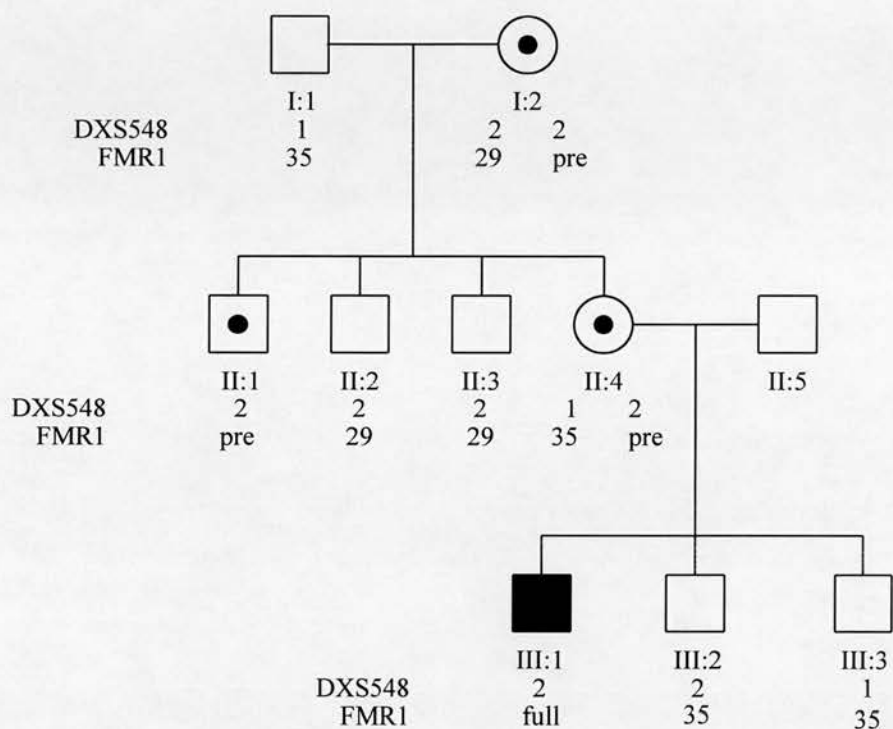


Figure 23. Pedigree of Family A showing results of *DXS548* and *FMRI* CGG repeat analysis. *DXS548* alleles are numbered arbitrarily with allele 1 being the largest of the two alleles seen in this family; the *FMRI* CGG repeat alleles are denoted as numbers of repeats except for the pre- and full mutations which are denoted as pre or full respectively. Symbol designations are as described previously.

As shown in Figure 23, a cross-over has occurred between *DXS548* and the fragile X locus in the meiosis which resulted in individual III:2. This child was predicted to be unaffected on the basis of a normal sized band on Southern blot analysis and a PCR allele in the normal range, but has inherited the *DXS548* allele which is associated with the fragile X mutation in this family. As the grandmother of the affected boy is homozygous for *DXS548*, phase for this marker is established from the grandfather of

the affected boy (I:1). Following identification of the full mutation in individual III:1, the mother II:4 opted for prenatal diagnosis in her subsequent pregnancies. In the first of these, a normal PCR allele of 35 repeats and normal sized fragments on Southern blotting were seen in the CVS DNA. However, the fetus had the same *DXS548* haplotype as the affected boy III:1. In the second of the prenatal diagnoses, the fetus was shown to have inherited the normal grandpaternal haplotype. Both pregnancies proceeded to term.

The physical distance between *DXS548* and *FRAXA* is reported to be 150kb (Riggins et al., 1992) and therefore the probability of a cross-over occurring in this interval is  $1.5 \times 10^{-3}$  (assuming that 150kb is approximately equivalent to a recombination frequency of 0.15%). Dreesen et al. (1994), however, have suggested that the genetic distance between these loci may be 0-6cM based on the data of Riggins et al. (1992) and they reported two recombinations in two fragile X families. On the basis of the *DXS548* results shown in Family A, individual III:2 would have been predicted to be affected in a prenatal diagnosis, although obviously this system would not be used alone. If Southern blot results were unobtainable and the laboratory had to rely on PCR methods alone, then the four systems (*FMRI* CGG, *DXS548*, *FRAXAC1* and *FRAXAC2*) should ideally be used together. The disadvantages of using the linked PCR systems is that an affected individual is required to obtain phase and in rare cases, CA repeats can “mutate” to larger alleles by the addition of one dinucleotide repeat.

Using linked markers for prenatal diagnosis of fragile X syndrome has one very serious drawback, however, which is that linkage cannot distinguish a premutation and a full mutation. The final prenatal diagnosis presented here describes a situation in which linked markers would have indicated an affected fetus.

### 2.3.3.3 Prenatal diagnosis of a premutation carrier male

#### Case report

The pedigree of the family (Family H) is shown in Figure 24. Individual II.12 was referred early in her first pregnancy, because of a history of fragile X syndrome. The family had been investigated cytogenetically in 1989, prior to the identification of the *FMRI* gene and as the fragile site had only been demonstrated in the affected male (III.1), individual II.12 and her three sisters were given low risks of being carriers. The affected girl III.3 was not tested at that time. The family was reanalysed by

- PCR across the repeat using primers **c** and **f** as described in **6.1.4.1**, and
- Southern blotting using StB12.3 with *HindIII* digested DNA (**2.1.3.19** and **6.1.2**, **6.1.3**).

The PCR and Southern blot results (Figures 25 and 26, pages 103 and 104) indicated that the dead father (I.1) must have been a normal transmitting male who had passed a premutation to each of his four daughters. The mother (I:2) had two normal CGG alleles, both of which had been passed to normal sons (Figures 24 and 25), providing confirmation that the father was a carrier.

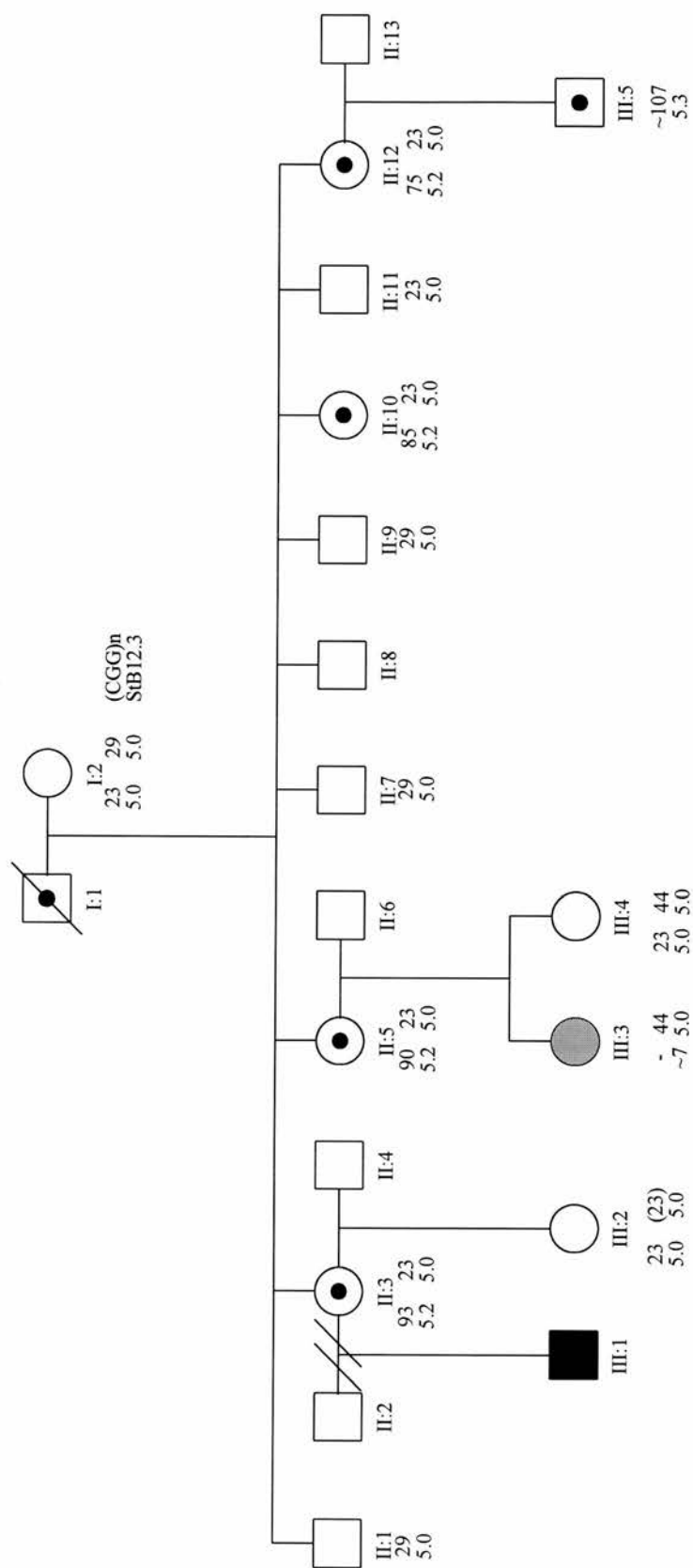


Figure 24. Pedigree of family H. Carriers of premutations are represented by symbols with a dot, the affected male is represented by a shaded black symbol, and the female carrier of a full mutation is represented as a shaded grey symbol. The CGG repeat number was estimated by comparison with a normal female control of 20 and 30 repeats, the premutation sizes were estimated by comparison with a size marker (*Msp*I digested pBR322 end labelled with  $\gamma$ - $^{32}$ P-ATP). The StB12.3 fragment sizes are in kb.

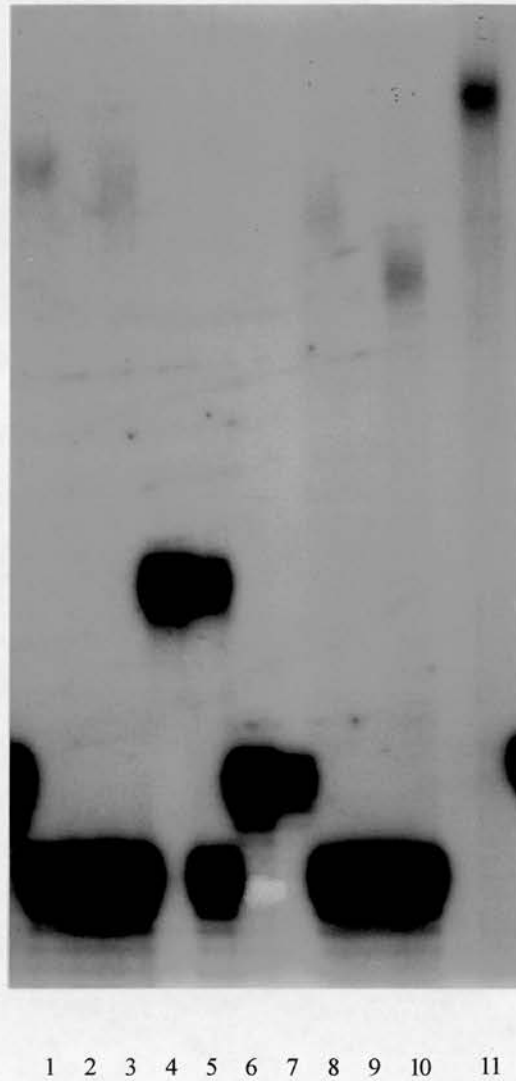


Figure 25. Results of PCR analysis on Family H showing premutation sized alleles in the four daughters of I:1: II:3, II:5, II:10 and II:12 (lanes 1, 3, 8 and 10). Lanes 2, 4, 5, 6, 7 and 9 contain PCR products from individuals III:2, III:3, III:4, II:7, II:9 and II:11. III:3 (lane 4) is an female with one allele of 44 repeats and a full mutation which does not PCR amplify. Lane 11 contains PCR product from fetal blood DNA (discussed later in this chapter).

A CV biopsy was carried out at 10 weeks gestation and DNA extracted as described in 6.1.1.2. The fetus was shown to be male by PCR at the PAB locus. Southern blotting of *Hind*III digested CV DNA showed an apparently homogeneous premutation (Figure 26, lane 10) similar in size to the mother's fragment (lane 9).

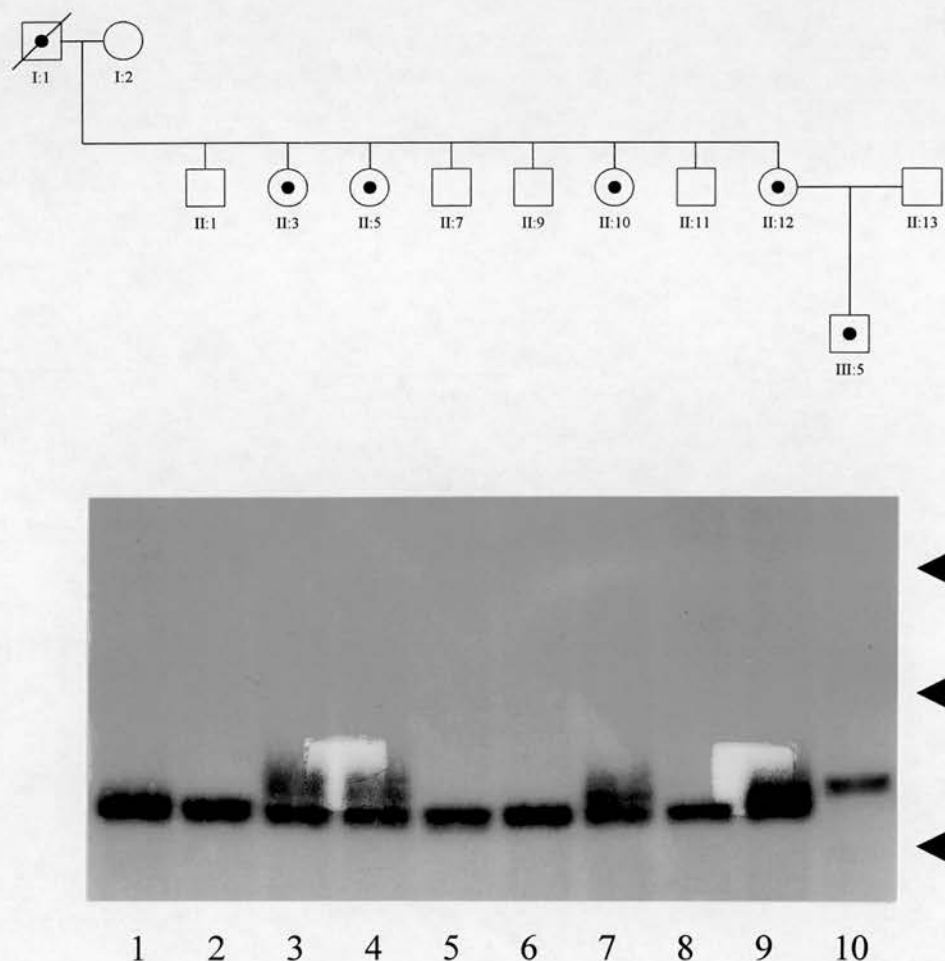


Figure 26. Southern blot analysis of family H showing premutations in all daughters of I:1 (lanes 3, 4, 7 and 9) and the male fetus of II:12 (lane 10), and normal sized fragments in all male offspring of I:1 (lanes 1, 2, 5, 6 and 8). *Hind*III digested DNA was probed with StB12.3. The arrows indicate the size marker (*Hind*III digested  $\lambda$  DNA): 9kb (top), 6.6kb (middle) and 4.4kb (bottom). Individuals are numbered as in Figure 24.



The PCR allele in the CV DNA was estimated to consist of 107 repeats which had increased slightly from the mother's premutation allele of an estimated 75 repeats (Figure 27).

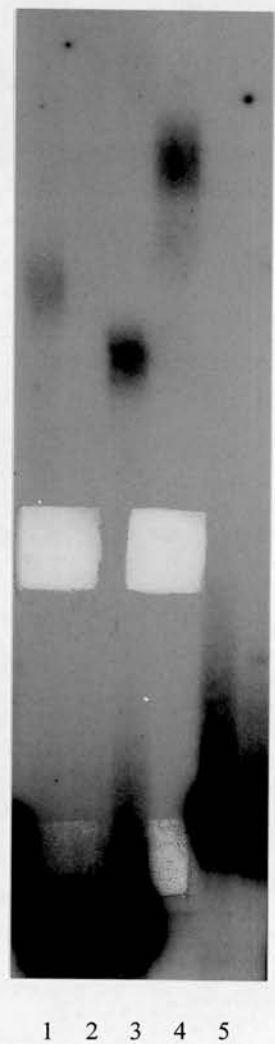


Figure 27. PCR analysis of CVS DNA showing slight increase in size from mother to fetus. Lane 1: individual II:10 (premutation carrier); lane 2: II:11 (normal male); lane 3: II:12 (mother of fetus III:5); lane 4: III:5 (CVS DNA); lane 5: (male control).

Cultured CV tissue showed a normal 46, XY karyotype with no evidence of fragile sites (cytogenetic analysis was carried out by Professor Christine Gosden, formerly of MRC Human Genetics Unit, Edinburgh).

The presence of a homogeneous premutation in CV DNA and a normal karyotype predicted that the fetus was a normal transmitting male and the pregnancy continued. However, at the time that this CVS was carried out (1993) evidence was accumulating which suggested that care was needed in the interpretation of results obtained on CVS tissue. Firstly, finding an apparently homogeneous premutation does not rule out the possibility of mosaicism for a premutation and a full mutation (which makes up only a small proportion of the somatic cell population and is therefore not detected by current methods). Mosaic males are generally not more mildly affected than males with only full mutations (Rousseau et al., 1994). Second, although evidence tends now to indicate that expansion from premutation to full mutation is a post-zygotic event and that mosaicism is established before chorionic villus sampling (usually around 10 weeks gestation), this information was not available at the time of the prenatal diagnosis carried out in family H. Third, the situation in extraembryonic tissues may be different to that in fetal tissues; this is certainly true for methylation of *FMRI*. All these factors were considered, and the patient was advised to have fetal blood sampling which was carried out at 18-19 weeks gestation.

Approximately 2 mls of fetal blood was obtained and was confirmed to be purely fetal by Kleihauer testing (carried out by Professor Christine Gosden). One ml was used for cytogenetic testing of the fragile site; 170 lymphocytes cultured under thymidylate stress failed to show any evidence of fragile sites and DNA was extracted from the other half as in 6.1.1.3. Southern blotting and PCR again showed a homogeneous premutation (Figures 28 and 29).

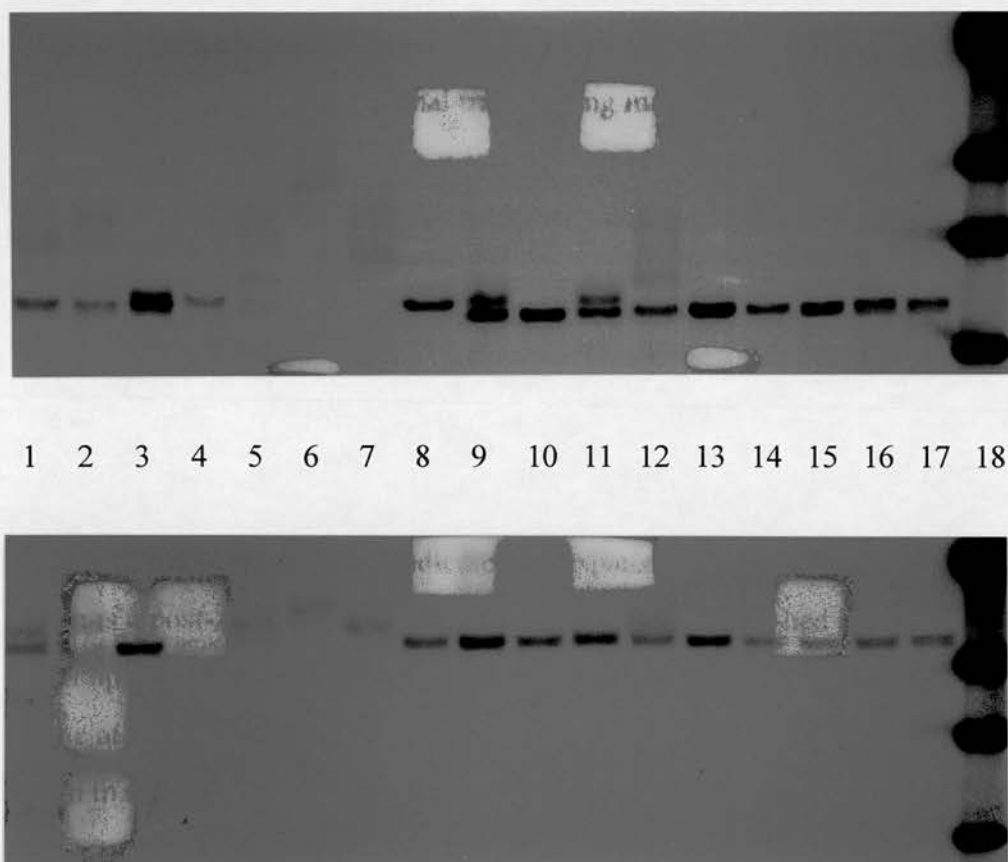
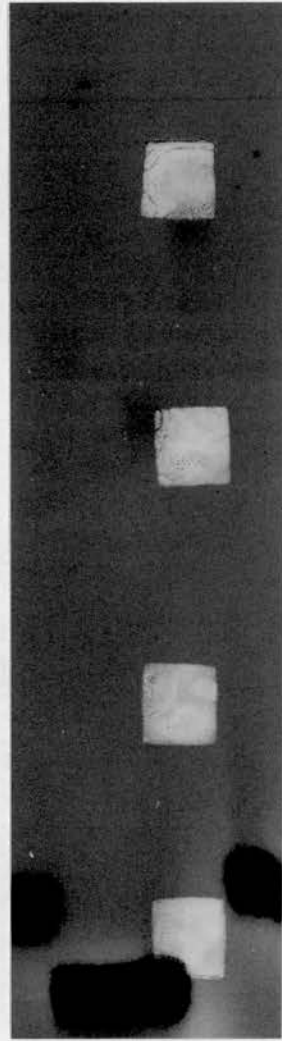


Figure 28. Southern blot analysis of fetal blood DNA. DNAs were digested with *Hind*III (top) or *Bgl*II (bottom) and probed with StB12.3. Lanes 1 and 2: full mutation carrier female controls; lane 3: mother of fetus; lane 4: fetal blood; lane 5: mosaic male control; lanes 6 and 7: full mutation male controls; lane 8 : NTM control; lanes 9 and 11: premutation carrying female controls; lane 12: full mutation carrying female; lanes 13-17: normal female and male controls. Marker (lane 18) is *Hind*III digested  $\lambda$  DNA. Bands shown here are 23kb (top), 9kb, 6.6kb, 4.4kb.



1 2

Figure 29. PCR analysis of fetal blood DNA. Lane 1: Mother of fetus (II:12); lane 2: fetal blood DNA. This PCR product is seen more clearly in Figure 25.

Methylation analysis of the *FMRI* CpG island was carried out by Southern blotting of fetal blood DNA digested with *EcoRI* and *BstZ1*. Normal methylation patterns consistent with a premutation were seen (not shown). From these data the patient was reassured that her fetus was normal.

At term, DNA was prepared from cord blood. A premutation was again seen on Southern blotting (Figure 30 and 31) and PCR analysis (Figure 32). When PCR products from amplification of CVS DNA and cord blood DNA were run side by side on the same gel, they were identical in size (not shown). The PCR product from cord blood DNA contains an allele which is the same size as the mother's normal allele. This may be due to transplacental bleeding during labour.

Cytogenetic analysis of the cord blood failed to show fragile sites in 100 cells analysed.

DNA analysis using linked markers could not be carried out in family H for two reasons. The affected boy (III:1) was not available, and in this case linkage results would have shown that the fetus had inherited the mutation carrying X chromosome from his mother. If this prenatatal diagnosis had occurred prior to the identification of *FMRI* this fetus might have been terminated on the basis of linkage results alone.

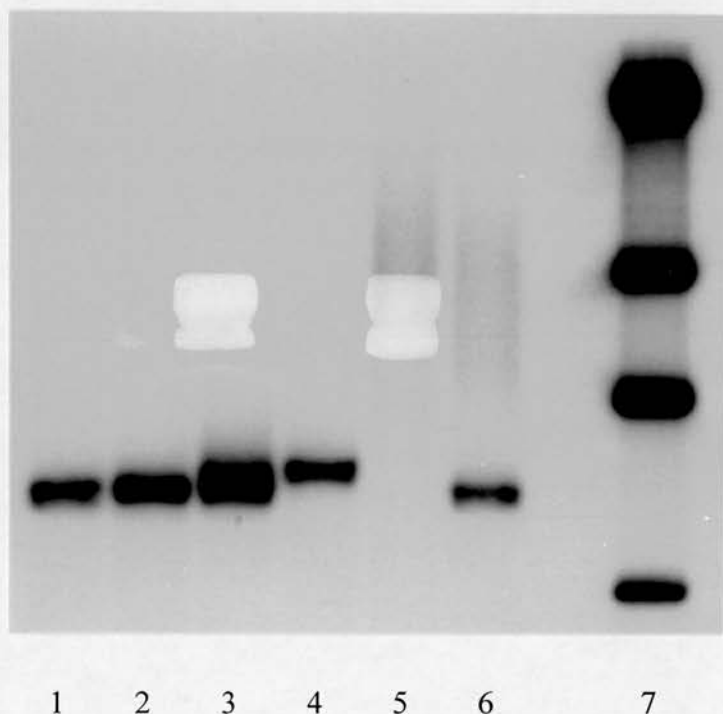


Figure 30. Southern blot of cord blood DNA digested with *Hind*III and probed with StB12.3. Lane 1: normal male control; lane 2: normal female control; lane 3: mother of baby; lane 4: cord blood DNA; lane 5: full mutation male control; lane 6: full mutation female control; lane 7: size marker (*Hind*III digested  $\lambda$  DNA: bands shown here are 23kb (top), 9kb, 6.6kb, 4.4kb).



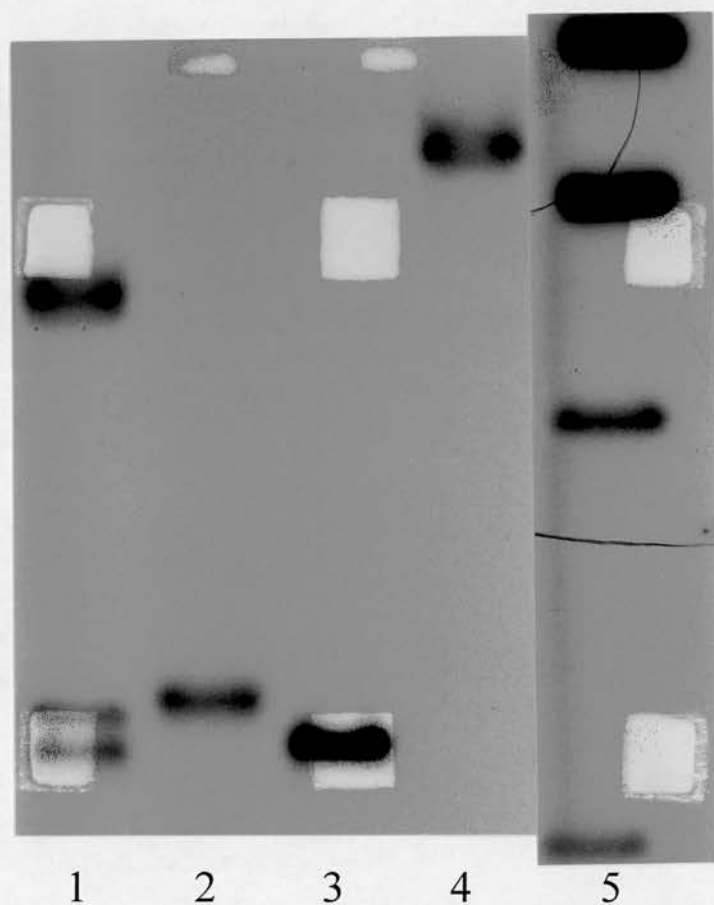


Figure 31. Southern blot of cord blood DNA digested with *Hind*III and *Eag*I and probed with StB12.3. Lane 1: mother of baby; lane 2: cord blood DNA; lane 3: normal male control; lane 4: full mutation male control; lane 5: size marker (*Hind*III digested  $\lambda$  DNA, 9kb, 6.6kb, 4.4kb and 2.2kb).



Figure 32. PCR analysis of DNA from cord blood. Lanes 1-3: normal controls; lane 4: mother of baby; lane 5: cord blood DNA. Although the premutations are very faint, there appears to be no further increase in size between mother's and baby's alleles.

### Experience gained from these prenatal diagnoses

The three cases described above have illustrated the variability which can occur in prenatal diagnostic situations. With experience of nine prenatal diagnoses for fragile X syndrome, I have learned that it is important to plan from the beginning how to handle each case so that the maximum information can be gained from the limited CV tissue. It would have been interesting, for example, (although not essential to the outcome), to have carried out methylation analysis in the twin pregnancy (2.3.3.1) and on the aborted material to determine if the twins had discordant methylation patterns. It may be interesting to analyse cell lines which were set up from the termination tissues to see if there are any differences in full mutation size and/or methylation after cell culture. Kruyer et al. (1994) described a pair of monozygotic female twins who had similar sized full mutations but the proportion of active and inactive X chromosomes carrying the mutation were very different in each twin and the phenotypes were correspondingly different.

The results of the prenatal diagnosis described in 2.3.3.1 were very informative in that the timing of expansion could be confined, in this case, to a very narrow interval during embryonic development i.e around day 5. A similar case was described by Antiñolo et al. (1996) who analysed monozygotic male twins who were concordant mosaics for a full mutation and an unmethylated premutation. The twins were monochorionic and monoamniotic which indicates a later form of twinning occurring between day 10 and 20 after fertilization. Since the separation of the gametic and somatic cell lineages occurs at approximately day 5, the timing of expansion in the twins described by Antiñolo et al. (1996) can be confined to between day 5 and day 20. The analysis of monozygotic twin pregnancies in fragile X syndrome is important for establishing the time at which a particular event occurs; twin studies have also shown that while expansion from premutation to full mutation occurs before twinning, methylation occurs much later (and probably in terms of weeks rather than days).

For the prenatal diagnosis discussed in **2.3.3.3** in which a male fetus with a premutation was predicted, the fetal blood sample was divided so that cytogenetic analysis could also be carried out. Although, at the time, it was reassuring to have a backup system, the blood sample was very small (<2ml) and half of it was clotted. The blood was collected into lithium heparin which in my experience does not give as high a yield of DNA as from EDTA anticoagulated blood and heparin (which binds to DNA) is thought to retard DNA during electrophoresis. In future prenatal diagnoses for fragile X syndrome, we would probably not use cytogenetic testing for the fragile site as a backup.

Fortunately, none of the prenatal diagnoses carried out in this laboratory predicted a full mutation carrying female which could potentially be clinically more problematic.

## 2.4 DISCUSSION

Although information on fragile X syndrome is continually emerging and evolving, it is now reasonably established that: a) expansion from premutation to a full mutation occurs post-zygotically and only after female meiosis, b) the stability of a CGG repeat is related not only to its length but to its composition, c) the presence of a full mutation in a female does not imply that she will be mentally retarded, nor is the length of full mutation repeat correlated with the degree of mental impairment and d) there are differences in the degree of somatic mosaicism and methylation between embryonic and extra-embryonic tissues. However, the following questions remain partly or fully unresolved:

Do the small changes in size of premutation from normal transmitting male to premutation carrying offspring occur meiotically or mitotically (post-zygotically)?

Does repeat expansion cause methylation at the *FMR1* CpG island?

Is there tissue specific variation in the size and/or methylation status of full mutations?

Are premutations in males associated with mental retardation?

### Is it time for prenatal screening for fragile X syndrome?

The idiosyncratic nature of the *FMR1* mutation and its behaviour upon transmission are probably mostly responsible for the fact that five years after the *FMR1* gene was identified, routine antenatal screening is not yet established in a U.K. genetic centre. As mentioned earlier, fragile X syndrome is an ideal candidate for prenatal screening because the sensitivity and specificity of the DNA test approaches 100%. Since all mothers of children with a full mutation have either a premutation or a full mutation, prenatal screening would detect all those mothers at risk of having a child with fragile X. However, there is not yet a reliable PCR method of detecting all carrier females and as described previously, if the criterion of two alleles differing by two or more repeats is used, then approximately 45% of women will require further testing by Southern blotting. Furthermore, Southern blotting cannot be carried out on a mouthwash sample so for this 45%, a blood sample would be required. There are

two routes by which DNA testing could be carried out in an antenatal setting. Either, a small blood sample could be collected at the first clinic appointment, DNA extracted by a boiled lysis method and PCR carried out across the CGG repeat. If the sample did not show two distinguishable alleles in the normal range, DNA would be extracted from the remainder of the blood sample for Southern blotting. The alternative approach would be to extract good quality DNA from the blood sample, carry out the *FMRI* PCR and, if necessary, Southern blotting. The first approach would probably be less time consuming and would also be cheaper (money would be saved on Proteinase K). For screening large numbers of samples, a non-radioactive method of detection would have to be used, either fluorescent PCR and electrophoresis on A.L.F. sequencing gels or silver staining of unlabelled PCR products. It is difficult to predict, without a pilot study, which method would be the most efficient and the most practical. However, funding for such a prenatal screening programme is not presently available.

#### The future for fragile X testing in Edinburgh

After an initial slow uptake by clinicians for the fragile X DNA test, the average rate of sample receipt has been 180 samples per annum, which is a considerable number for a test with a low detection rate (approximately 1%). This is very low for a procedure which is labour intensive and costly (each DNA test starting from blood is estimated to cost between £5.50 and £6.00 for reagents alone). Each sample received in this laboratory is analysed by Southern blotting as well as PCR and in order to improve efficiency, samples are batched into multiples of sixteen (the capacity of an agarose gel). The ratio of males to females referred for testing is approximately 3.3:1 and therefore, on average, each gel includes DNA from three females (taking up two lanes for a single and double digest) and DNA from ten males (one digest only). Depending upon the rate at which samples are received and the proportion of females referred for testing, the time from sample receipt to final result ranges from 1-2 weeks to 8 weeks (obviously, a sample arriving at the end of a batch will have a shorter reporting time). In practice, more than 70% of fragile X results were reported within six weeks.



It may be an appropriate time to review DNA testing for fragile X syndrome and assess whether we should continue as previously or progress to more economical and more efficient methods of testing. There are two areas in which improvements could be made:

- 1) If samples were tested by PCR and then Southern blotting carried out only if necessary i.e. if a female sample was homozygous or a male sample gave no result. Using the data presented in this chapter, 11% of all the samples received failed to give a satisfactory diagnostic result by PCR analysis.
- 2) If there was more discriminatory referral of patients for testing the proportion of negative test results could be reduced from the present figure of 98-99%. A prospective survey, organised by the BSHG, is currently underway to establish which clinical criteria have been associated with a positive DNA test result. A simple scoring system will be developed and DNA testing only carried out on patients with an appropriate score. It is important that patients with mental retardation are still referred for cytogenetic testing for other chromosomal abnormalities, however. Preliminary data from the United States suggests that it may be possible to reduce the number of fragile X tests by over 50% without missing any true affected cases.

### **3. Mapping of a non-specific mental retardation gene to Xq12-Xq21.**

#### **3.1 INTRODUCTION**

When a child presents with developmental delay without any obvious clinical features, the paediatrician will generally want to exclude the most common causes of mental retardation by a series of tests. These may include cytogenetic analysis to rule out chromosomal abnormalities, biochemical tests to exclude metabolic disorders and DNA tests to rule out genetic diseases. As the prevalence of fragile X syndrome is approximately 1 in 1500 with a variable phenotype, many clinicians will refer a child for DNA testing for this disorder on the basis of mental retardation alone. However, as described earlier, only 1-2% of children with developmental delay will subsequently be shown to have a full *FMR1* mutation. For the majority of cases of childhood mental retardation, a specific defect will never be found. Nevertheless, fragile X syndrome is one of the most common disorders for which a simple diagnostic test is widely available.

A family (Family I) is presented in this chapter, in which a boy was first investigated for fragile X syndrome because of developmental delay and also because of behavioural problems which were considered to be typical of fragile X syndrome. DNA testing specific for fragile X syndrome showed that the boy had a PCR CGG repeat allele in the normal range and a normal fragment on Southern blotting. In spite of this, it was still thought to be highly probable that the behavioural phenotype represented fragile X syndrome. Since it was possible that another form of *FMR1* mutation other than the CGG repeat expansion was responsible for the phenotype, linkage analysis using the polymorphic CGG repeat as a marker was carried out on the proband and other family members (including the proband's cousin and two maternal uncles who were also mentally retarded). LOD scores were negative with *FMR1*. The inheritance of the mental retardation in the family was presumed to be X-linked because only males were affected and there was no male to male

transmission. As a result of obtaining a negative LOD score with *FMRI*, further linkage analysis in this family, using highly polymorphic microsatellite markers covering all of the long arm of the X chromosome, and a small region in proximal Xp, was carried out. Positive LOD scores above +2 were found with markers in the proximal long arm (Xq13-Xq21) within a 10cM interval. At least eight other non-specific mental retardation conditions map within this interval.

### 3.2 FAMILY STUDIES

The family (Family I) described in this chapter is shown in Figure 33.

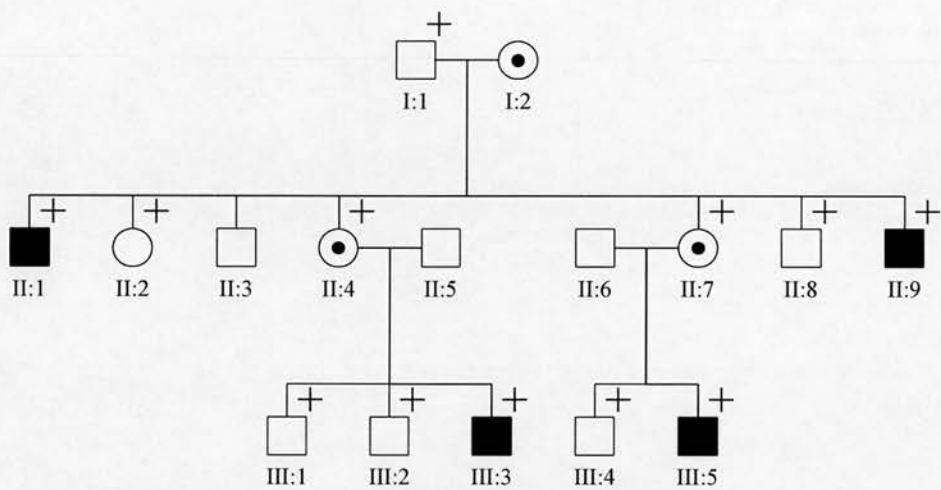


Figure 33. Pedigree of family I. Obligate carrier females are represented by symbols with a dot, males with mental retardation are represented by filled black symbols. Blood was obtained from those individuals marked with a cross.

#### 3.2.1 Case reports

The proband The proband in family I (III:5), originally presented as a mildly dysmorphic boy with developmental delay and aggressive behaviour. He was born in 1982 after a normal pregnancy and delivery, although he was of relatively low birth weight (2.8kg) and had a small head (OFC well below the 10th centile) in the early neonatal period. His motor development was slightly delayed; he did not walk until

19 months of age. He was described as having an unusual facies but “within normal limits” and he had marked epicanthic folds and full lips. He had a slight systolic murmur (also present in his mother) which was initially thought to be a ventricular septal defect (VSD) but later investigations when he was 11 years of age showed that his heart anatomy was normal (the VSD was presumed to be closing). There was no family history of congenital heart disease. A CT scan was performed at the age of four because of a single epileptic fit; the scan was reported as normal with “*no cause for this patient’s focal fit identified*” and also demonstrated that there was no definitive lesion within the CNS to account for his mental retardation. An EEG showed a “*probable excess of slow wave activity but no focal or paroxysmal activity*” which was thought to be consistent with the boy’s overall developmental delay. At the age of six, he was described as hyperactive with autistic features, atrocious language skills and unintelligible speech.

Other affected family members A cousin of the proband is also mentally retarded (III:3) and was reported to “*look the same and behave the same*” as the proband. He also attends a special school, has delayed speech and is unable to read or write.

Two maternal uncles of the proband (II:1 and II:9) are also mentally retarded with delayed speech; both attended special schools. The uncle (II:9) had a significantly small head as a child with prominent epicanthic folds but no other significant physical abnormalities. A third maternal uncle (II:8) may have been slightly dull intellectually although he attended normal schools and has four children.

Both obligate carrier females (II:4 and II:7) are of normal intelligence.

### 3.3 METHODS

#### 3.3.1 DNA and cytogenetic analysis

Blood was obtained from family members marked on the pedigree (Figure 33) with a cross and DNA was extracted as in 6.1.1.1. In order to exclude fragile X syndrome as the cause of the mental retardation in the family, DNA analysis was carried out on the proband (III:5) and his mother (II:7) by PCR across the *FMRI* CGG repeat.

Alleles in the normal range were seen in both cases. Cytogenetic analysis on the proband (carried out by the Lothian Area Cytogenetic Service at the Royal Hospital for Sick Children) showed a normal karyotype with no evidence for FRAXA or other fragile sites.

The easiest way of finding if an *FMRI* mutation other than the CGG repeat expansion was present in the proband was to use the polymorphic repeat as a marker for linkage analysis of the whole family. If linkage could be excluded then it would not be necessary to search the *FMRI* gene for a mutation. The results are shown in Figure 34 (page 125). Even without formal linkage analysis, it was obvious from inspection of the pedigree that there was no linkage between the *FMRI* locus and the MR locus in family I. For this reason, I undertook a search for linkage to other regions of the X chromosome using polymorphic microsatellite and VNTR markers (heterozygosities generally above 0.7). The details of each marker are shown in Table 13. A total of 31 markers were analysed, although only 23 of these were informative in the family (these are shown on the pedigree in Figure 35, page 126). PCR markers were analysed covering all of Xq and the proximal region of Xp.

All the markers shown in Table 13 (page 124), apart from the *FMRI* CGG repeat, were analysed by PCR with one fluorescent primer using conditions as described in 6.1.4 and annealing temperatures as shown in Table 13. The *FMRI* CGG repeat was analysed as in 6.1.4.1 using primers **c** and **f**, and PCR products were electrophoresed in a 5% denaturing sequencing gel. The non-radioactive PCR products were analysed on the automated laser fluorescent sequencer (A.L.F., Pharmacia) in 6%

denaturing polyacrylamide gels. A typical result of a marker analysed in this way (*HPRT*) is shown in Figure 36 (page 127). For each marker analysed the largest allele in the family was denoted as allele 1 and the smallest as 3.

The following markers were also analysed in this family but were uninformative: *DXS178*, *DXS237*, *COL4A5*, *DXS1231*, *DXYS1*, *DXS135* and *CHM* (CA repeat within the choroideremia gene).

### 3.3.2 Linkage analysis

Two-point linkage analysis was carried out using the MLINK component of the LINKAGE program package version 5.2 (Lathrop and Lalouel 1984). An arbitrary mutation rate of  $1 \times 10^{-5}$  was chosen. If male and female mutation rates are equal and the XLMR condition segregating in this family is reproductively lethal, this corresponds (in a population at equilibrium) to a gene frequency of  $3 \times 10^{-5}$  (Ott 1991). These figures were used for the MLINK calculation. Complete penetrance for the disease was assumed. The results of the pairwise analysis are shown in Table 14 (page 131).

Multipoint analysis was also carried out using the LINKMAP component of FASTLINK using genetic distances as shown in Figure 37 (page 130). If the genetic distance between two markers was zero, only one of the markers was included in the multipoint analysis. Sixteen of the 23 informative markers were subsequently used for the LINKMAP analysis. As the relationship between genetic distance and recombination fraction is only linear below  $\sim 10\text{cM}$ , genetic distances greater than this were converted into recombination fractions using the Kosambi mapping function. According to Ott (1991), this map function is preferable to the Haldane map function because it takes into account the effect of interference and therefore produces more realistic map distances.

Because of the computer memory limitations on the number of haplotypes (a maximum of 64 haplotypes, including the disease locus) which can be analysed at



one time on a stand-alone PC, the LINKMAP analysis was carried out, via Telnet, on a mainframe computer at the Human Genome Mapping Project Resource Centre. The method used was that recommended by Terwilliger and Ott (1994) which involves “sliding” groups of four loci and the disease locus down the map and analysing the disease only in the middle interval (i.e. with two flanking markers on each side). This was repeated until all the markers had been analysed. Five evaluations were computed within each test interval unless the interval was greater than 10cM when ten were calculated. For the purposes of the multipoint analysis (and because of HGMP restrictions in the LINKAGE programs), the mutation rates were set at zero. The linkage report program (LRP) was then used to obtain multipoint LOD scores which were plotted against genetic distance (using the marker *DXS1204* as a starting point) and the resulting graph is shown in Figure 38 (page 132).

Location	Locus	Reference	Primer sequences (5'-3')	Anneal temp.
Xp11.22	<i>DXS1204</i>	Gyapay et al. (1994)	F ATGAACCCCTTAACCTCATTTAGCAGG R AGCNTGCACCAACATGCC	55°C
Xp11.21	<i>DXS991</i>	Gyapay et al. (1994)	F ACTTCAACCACAGAAGCCTC R ATCATTTGAGCCAATTCTCC	55°C
Xq12	<i>AR</i>	Sleddens et al. (1992)	F TCCGCGAAGTGATCCAGAAC R CTTGGGGAGAACCATCCTCA	55°C
Xq12	<i>DXS339</i>	Zonana et al. (1992)	F ATGAAATAGCCCAGTACTCC R TCTGCTATAACCACACCATC	54°C
Xq12	<i>DXS106</i>	Fairweather et al. (1993)	F CCTGTACTCCTAGGTATTTGT R AGAGAGCCAAATCAAGAATGC	60°C
Xq13.1	<i>DXS453</i>	Rider et al. (1993)	F GCCCCTACCTTGGCTAGTTA R AACCTCAGCTTATACCCAAG	55°C
Xq13.3	<i>DXS566</i>	Porteous et al. (1992)	F CATTGCCAGAATATATGAAACAT R ACTTGTCAAATATGGCTCATA	55°C
Xq13.3	<i>PGK1</i>	Graeber et al. (1992)	F CCACAGCAAAAAACAAACAGA R GTAGAAGGGTAAGCATTATT	58°C
Xq21.1	<i>DXS986</i>	Gyapay et al. (1994)	F CCTAAGTGCTCATCATCCCA R AGCTCAATCCAAGTTGCTGA	55°C
Xq21.1	<i>DXS1197</i>	Gyapay et al. (1994)	F GCATGTTTATATCAGCAGAATTTG R TCCAACCTCCATCTAGTTTGC	55°C
Xq21.1	<i>DXS995</i>	Gyapay et al. (1994)	F AAGGGGCTGCTGATGATTAT R AATGCGTTCCCCAAATGT	61°C
Xq21.33	<i>DXS3</i>	Stanier et al. (1991)	F AATACATAGGTGTATTGTGACC R CCACCTCTCTGAAAGTGTGT	55°C
Xq21.33	<i>DXS1203</i>	Gyapay et al. (1994)	F CCTGAATTTCCCCAGC R TCCCACCTGCCAACCTC	55°C
Xq21.33	<i>DXS990</i>	Gyapay et al. (1994)	F AGCTATATGACCAGTACAAACATAC R GACAGAAGGGACATCAACTC	63°C
Xq22.3	<i>DXS1106</i>	Gyapay et al. (1994)	F TATGAGAACTCCCTAAACAAA R TGATGCACCAAATACCA	55°C
Xq22.3	<i>DXS1105</i>	Gyapay et al. (1994)	F TGTGAGGTTCAAGCCCC R GTTTCCTGATTACCATTTAGAC	60°C
Xq23	<i>DXS1059</i>	Gyapay et al. (1994)	F CCAGGAGATTGAGAGTGGCT R GGACAACCCGTCGTGG	55°C
Xq23	<i>DXS1212</i>	Gyapay et al. (1994)	F TGGAAGCATGAGAAATCACATCCT R TGGCATTACAAGCCCTCAAGTC	65°C
Xq26.1	<i>HPRT</i>	Edwards et al. (1992)	F ATGCCACAGATAATACACATCCCC R CTCTCCAGAATAGTTAGATGTAGG	60°C
Xq26.2	<i>DXS1062</i>	Gyapay et al. (1994)	F GAGATGTGTGACCTTGAGCACT R GTTGCTGTTAAGCACTTTGAATC	55°C
Xq27.3	<i>FMRI</i>	Fu et al. (1991)	F GCTCAGCTCCGTTTCGGTTTCACTTCCGGT R AGCCCCGCACTTCCACCACCAGCTCCTCCA	65°C
Xq28	<i>RGCP</i>	see Table caption	F TCCAGGTGTTGCCATTGCAG R TGGGAGGACAGTCTCTCCAG	57°C
Xq28	<i>DXS1108</i>	Freije et al. (1992)	F GTGAATTCATCATATGTGATTTC R ACTAGGCGACTAATACAGTGGTGC	55°C

Table 13. DNA markers analysed by PCR in Family I. Chromosome locations were obtained from Willard et al. (1994) and annealing temperatures for each primer pair were either as published or calculated from the sequence. Primer sequences for the *RGCP* CA repeat were obtained from Patrick Willems, University of Antwerp.

### 3.4 RESULTS

#### 3.4.1 DNA analysis

##### Preliminary results using PCR across the *FMRI* CGG repeat

The results of this analysis are shown in Figure 34.

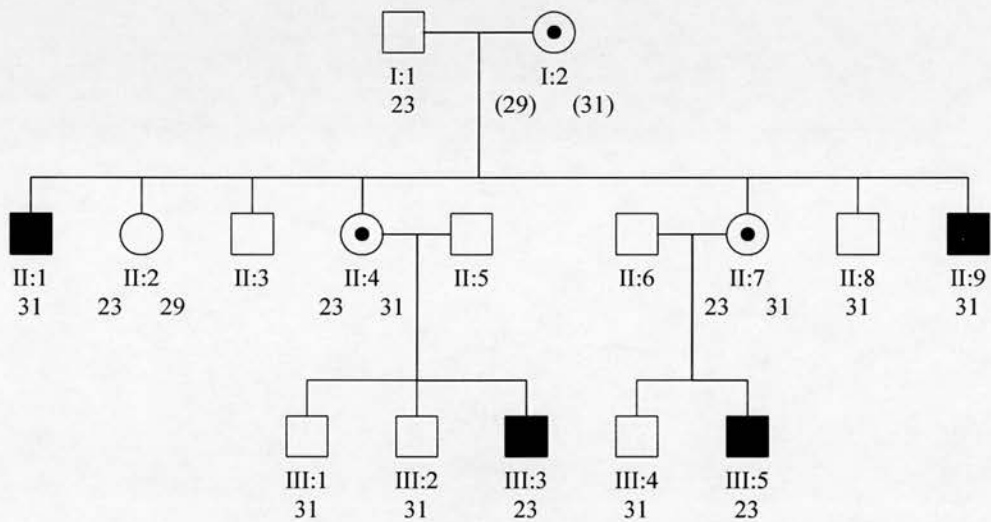


Figure 34. Results of *FMRI* CGG PCR analysis in family I. The CGG repeat number is shown below each symbol (the haplotype for individual I:2 is inferred from her offspring).

The affected and unaffected brothers in generation II have inherited the same maternal allele, and the two affected boys in generation III have both inherited the normal grandpaternal allele. The two-point LOD score for *FMRI* and the MR locus was  $-\infty$  at  $\theta = 0$  (see Table 14, page 131).

The 23 markers which were subsequently found to be informative in this family i.e. for which individuals II:4 and II:7 were heterozygous, are shown on the pedigree in Figure 35. When linkage to the proximal long arm was found, the density of markers analysed in that region was increased in order to define the interval more specifically, although the coverage depended upon the availability and informativeness of the markers in the family.

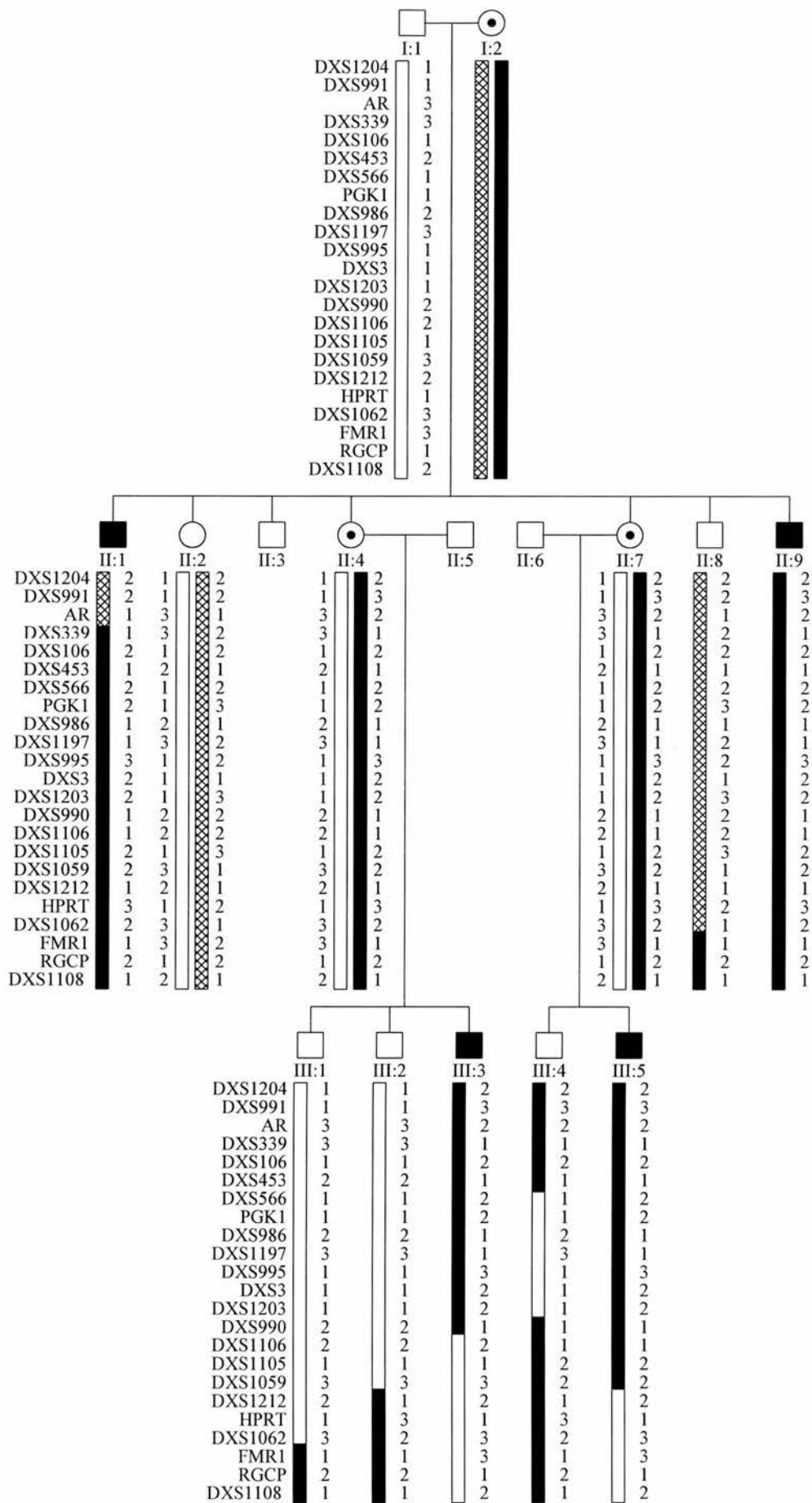


Figure 35. Pedigree of Family I showing results of DNA analysis. Markers are shown in chromosome order (Xp-Xqter). Symbol designations are as described in Figure 33.

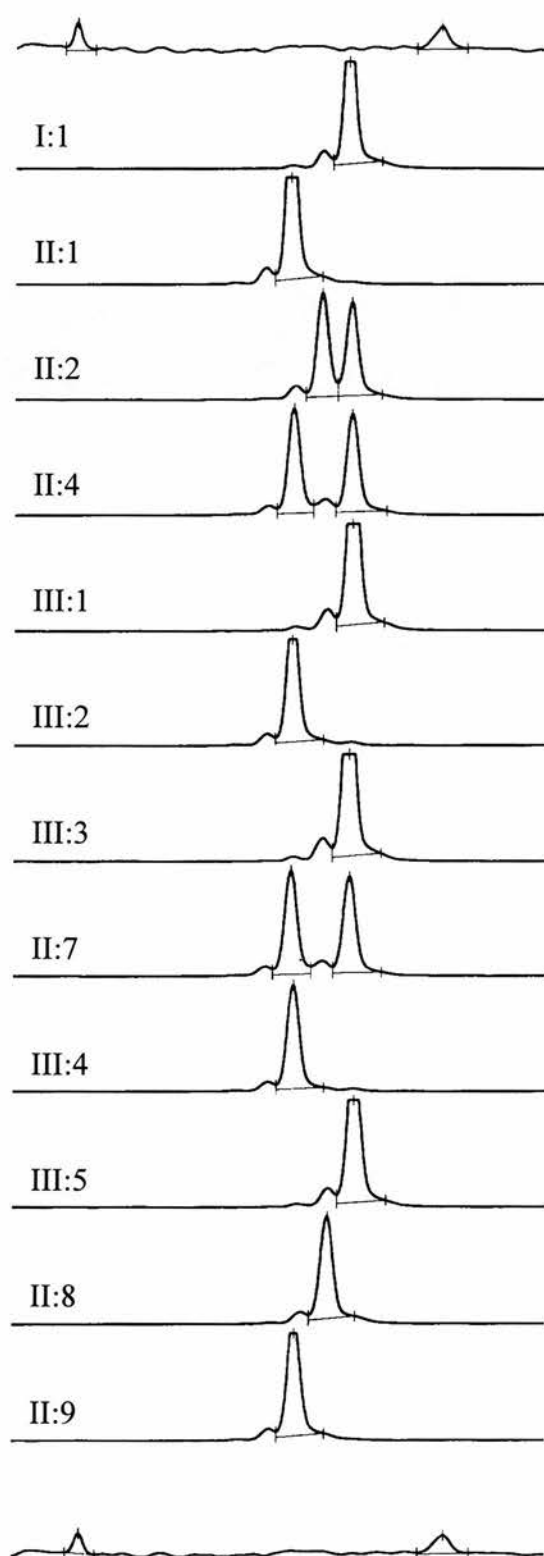


Figure 36. Results of analysis of the VNTR marker *HPRT* on the A.L.F. sequencer. The largest allele is arbitrarily denoted as 1 and the smallest allele as 3. The marker (described in 6.1.4c) shown in the first and last lanes consists of peaks at 250bp (left) and 300bp. The numbers on the left of each lane indicate the individual's identifier as shown in Figure 35.

In Figure 35, the three different shaded bars represent the three grandparental X chromosomes (black represents the affected haplotype). Alleles are numbered as 1 being the largest allele and 3 being the smallest allele seen in the family. As the haplotype for I:2 is unknown, the pedigree is drawn as if the individuals in generation II (apart from II:8) have inherited a non-recombinant X chromosome from their mother. The pedigree could equally have been drawn with only II:8 being non-recombinant and his siblings as recombinants for the markers in Xq27.3-Xqter (*FMR1* to *DXS1108*).

The bars in Figure 35 indicate that if the clinical status for each individual is as shown, then the only region shared by affected males but not shared with unaffected males is the region between the markers *DXS453* and *DXS990*. Linkage analysis was carried out to confirm this observation.

### 3.4.2 Linkage analysis

The linkage report program (LRP) was used to produce a two-point LOD score table from the two-point analysis and this is shown in Table 14. Positive LOD scores were obtained with the markers extending from *DXS566* to *DXS1203*. The maximum LOD score of 2.71 at a recombination fraction of zero was obtained with several markers (*PGK1*, *DXS1197*, *DXS995*, *DXS3* and *DXS1203*) producing a plateau of scores at 2.71. Two other positive LOD scores of 1.49 were obtained with *DXS566* and *DXS986*, the latter of which lies between two markers which gave scores of 2.71. Although this could be due to polymorphic differences in these markers, inspection of the pedigree shows that one of the key individuals (obligate carrier I:2) is homozygous for these markers (albeit inferred) and therefore this would be expected to reduce the LOD score. Two-point linkage analysis suggests, therefore, that the MR gene in Family I, is situated between *DXS566* to *DXS1203* (a distance of 6.8cM) although the precise limits may extend on either side to the markers *DXS453* and *DXS990* (10.1cM). In order to define the interval more specifically, multipoint linkage analysis was carried out using the genetic distances shown in Figure 37 (page 130) and the results of this analysis are shown graphically in Figure 38 (page 132).



Although the output of the LRP program from LINKMAP results in location scores, Terwilliger and Ott (1994) recommend converting these to multipoint LOD scores (by dividing each location score by  $2\ln(10)$ ) to provide a more meaningful comparison between two-point and multipoint analyses.

<i>Xpter</i> ↑	cM	θ
<i>DXS1204</i>	3.1	0.031
<i>DXS991</i>	0.5	0.005
<i>AR</i>	3.9	0.039
<i>DXS339</i>	0.0	
( <i>DXS106</i> )	2.3	0.023
<i>DXS453</i>	2.4	0.024
<i>DXS566</i>	0.0	
( <i>PGK1</i> )	0.0	
( <i>DXS986</i> )	0.0	
( <i>DXS1197</i> )	0.0	
( <i>DXS995</i> )	6.8	0.068
<i>DXS3</i>	0.0	
( <i>DXS1203</i> )	0.9	0.009
<i>DXS990</i>	6.2	0.062
<i>DXS1106</i>	7.0	0.070
<i>DXS1105</i>	2.0	0.020
<i>DXS1059</i>	16.8	0.162
<i>DXS1212</i>	11.3	0.111
<i>HPRT</i>	0.0	
( <i>DXS1062</i> )	28.7	0.259
<i>FMRI</i>	12.2	0.12
<i>RGCP</i>	2.1	0.021
<i>DXS1108</i> ↓ <i>Xqter</i>		

Figure 37. Genetic distances between informative markers analysed in Family I. Figures on the left are in cM and were taken from Fain et al. (1995). For the LINKMAP analysis these genetic distances were converted from cM to recombination fractions (shown on the right ) using the Kosambi mapping function [ $\theta = \frac{1}{2} \tanh(2x)$ ] where  $x$  is the distance in Morgans.

The marker *RGCP* is not listed by Fain et al. (1995) but is known to lie between *DXS15* and *F8C* (Willard et al., 1994), both of which are on the Fain map at the same genetic location. Markers in brackets were excluded from the LINKMAP calculation either because the genetic distance between two adjacent loci was zero.

LOD score at recombination fraction ( $\theta$ ) of:									
Locus	0.0	0.01	0.05	0.1	0.2	0.3	0.4	$Z_{\max}$	$\theta_{\max}$
<i>DXS1204</i>	$-\infty$	-0.52	0.11	0.32	0.42	0.37	0.22	0.42	0.19
<i>DXS991</i>	$-\infty$	-1.33	-0.07	0.34	0.54	0.46	0.25	0.54	0.19
<i>AR</i>	$-\infty$	-1.33	-0.07	0.34	0.54	0.46	0.25	0.54	0.19
<i>DXS339</i>	$-\infty$	0.67	1.21	1.30	1.14	0.80	0.37	1.30	0.10
<i>DXS106</i>	$-\infty$	-0.52	0.11	0.32	0.42	0.37	0.22	0.42	0.19
<i>DXS453</i>	$-\infty$	-0.52	0.11	0.32	0.42	0.37	0.22	0.42	0.19
<i>DXS566</i>	1.49	1.47	1.39	1.28	1.02	0.73	0.40	1.49	0.0
<i>PGK1</i>	2.71	2.67	2.49	2.25	1.74	1.17	0.54	2.71	0.0
<i>DXS986</i>	1.49	1.47	1.39	1.28	1.02	0.73	0.40	1.49	0.0
<i>DXS1197</i>	2.71	2.67	2.49	2.25	1.74	1.17	0.54	2.71	0.0
<i>DXS995</i>	2.71	2.67	2.49	2.25	1.74	1.17	0.54	2.71	0.0
<i>DXS3</i>	2.71	2.67	2.49	2.25	1.74	1.17	0.54	2.71	0.0
<i>DXS1203</i>	2.71	2.67	2.49	2.25	1.74	1.17	0.54	2.71	0.0
<i>DXS990</i>	$-\infty$	0.67	1.21	1.30	1.14	0.80	0.37	1.30	0.10
<i>DXS1106</i>	$-\infty$	-1.33	-0.07	0.34	0.54	0.43	0.19	0.54	0.19
<i>DXS1105</i>	$-\infty$	-1.33	-0.07	0.34	0.54	0.43	0.19	0.54	0.19
<i>DXS1059</i>	$-\infty$	-1.33	-0.07	0.34	0.54	0.43	0.19	0.54	0.19
<i>DXS1212</i>	$-\infty$	-6.39	-3.72	-2.54	-1.38	-0.74	-0.31	0.0	0.5
<i>HPRT</i>	$-\infty$	-5.31	-2.63	-1.57	-0.67	-0.31	-0.16	0.0	0.5
<i>DXS1062</i>	$-\infty$	-5.31	-2.63	-1.57	-0.67	-0.31	-0.16	0.0	0.5
<i>FMRI</i>	$-\infty$	-9.21	-5.18	-3.47	-1.87	-1.01	-0.45	0.0	0.5
<i>RGCP</i>	$-\infty$	-7.11	-4.94	-3.49	-1.98	-1.11	-0.48	0.0	0.5
<i>DXS1108</i>	$-\infty$	-7.11	-4.94	-3.49	-1.98	-1.11	-0.48	0.0	0.5

Table 14. Results of two-point LOD scores.  $Z_{\max}$  is the maximum LOD score and  $\theta_{\max}$  is the recombination fraction at which the  $Z_{\max}$  was obtained.

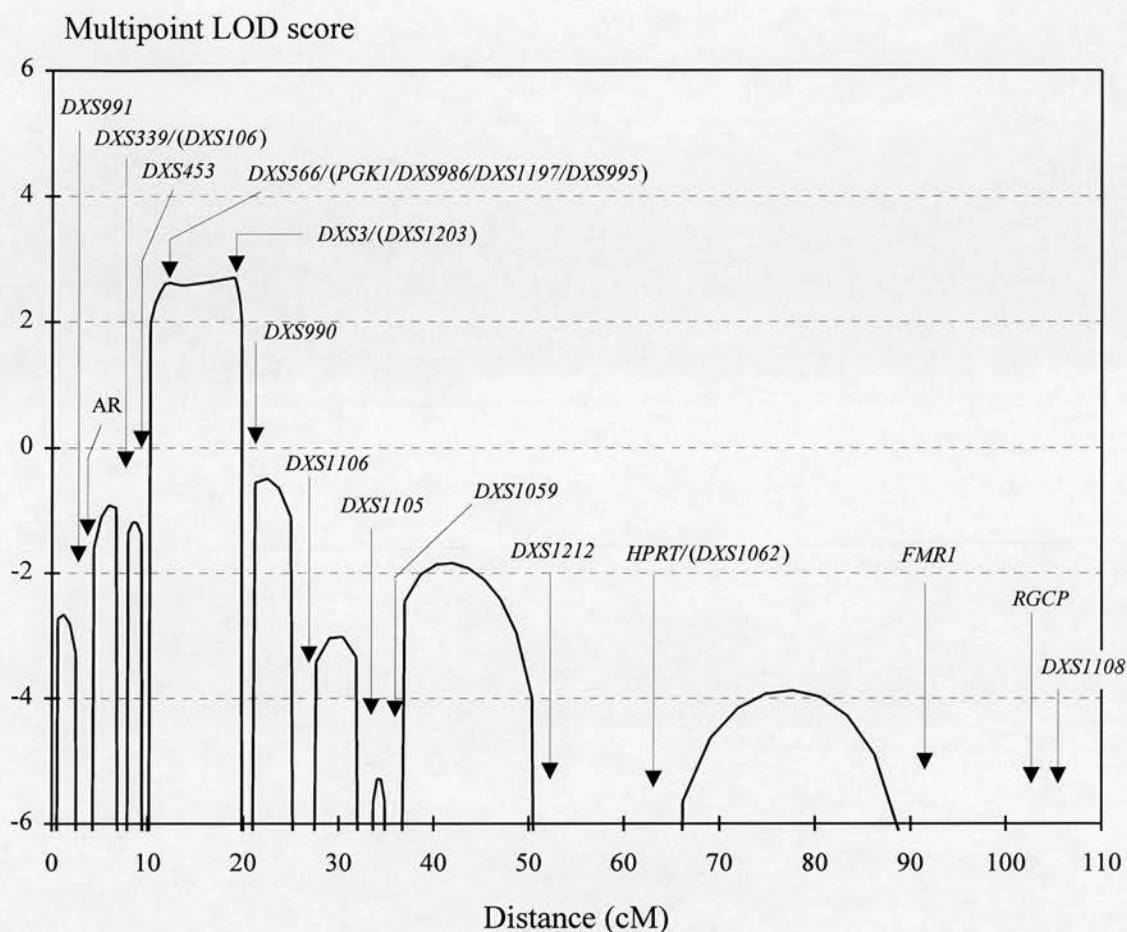


Figure 38. Results of the LINKMAP analysis in Family I. The marker *DXS1204* (not shown) was used as the starting point (0cM). Markers in brackets were not included in the LINKMAP analysis because the genetic distances between that marker and the adjacent marker was zero. The location scores produced by LRP were converted to multipoint LOD scores by dividing by  $2\ln(10)$  as described in Terwilliger and Ott (1994). LOD scores produced by this “sliding” method are not strictly comparable to a complete 16-point multipoint analysis. Furthermore, the odd shape of the curve in the region of the maximum LOD scores may be a consequence of carrying out this type of analysis. Alternatively, it may represent the overlap of two curves (one each for *DXS566* and *DXS3*) which show no recombination with the disease. The maximum multipoint LOD score obtained was 2.71 with the marker pair *DXS3/DXS1203*. Mutation rates were set at zero for this analysis and an arbitrary cut-off point of -6 was used for the y axis.

### 3.5 DISCUSSION

The mental retardation condition segregating in this family is thought to be a non-specific form of XLMR with global developmental delay. The only obvious clinical features observed in two out of four affected males were low birth weight, microcephaly in the neonatal period, and prominent epicanthic folds. The two boys in generation III are slightly dysmorphic. Although these males do not appear normal, the clinical features are so bland that the mental retardation condition affecting the males in this family is presumed to be non-specific (MRX) rather than syndromic (MRXS). Two-point linkage analysis using highly polymorphic X chromosome markers has placed the locus for this MRX condition to a 10cM region in Xq13-Xq21 (between *DXS453* and *DXS990*). Multipoint linkage analysis in this family, however, has not narrowed the region further than 10cM.

This region of the X chromosome contains a cluster of MRX loci which are shown in Table 15 (extracted from Table 5, Chapter 1).

Name	Locus	Description	Reference
MRX1	Xp11.4-q21.31	Mental retardation only	Suthers et al., (1988)
MRX4	Xp11.22-q21.31	Speech delay, learning disability	Arveiler et al., (1988a)
MRX5	Xp21.1-q21.3	Hyperactive behaviour, speech delay	Samanns et al., (1991)
MRX7	Xp11.4-q21.3	Mental retardation only	Jedele et al., (1992)
MRX8	Xp11.3-q21.22	Mental retardation only	Schwartz et al., (1992)
MRX12	Xp21.3-q21.1	Hypotelorism, large ears	Kerr et al., (1992)
MRX13	Xp22.3-q21.22	Large ears	Kerr et al., (1992)
MRX17	Xp11-q21.3	Mental retardation only	Gedeon et al., (1994)

Table 15. Non-specific XLMR conditions which map in the same region as the XLMR condition in Family I.

Linkage analysis in families with non-syndromal forms of XLMR is hampered by the fact that the families are usually small and the phenotype of affected males is either

very subtle or absent. For this reason, families cannot usually be combined to increase LOD scores, and many localisations are based on LOD scores barely reaching +2. The linkage based localisations of the MRX conditions listed in Table 15 cover large parts of the X chromosome (in some cases as much as 50%, corresponding to genetic distances of 80-90cM) and therefore comparison of these loci and the MR condition in Family I is probably not very helpful in determining whether this is the same condition as a previously described condition. Furthermore, the unaffected males in Family I were not examined and therefore we cannot tell whether the subtle dysmorphic facial features seen in the two males in generation III are peculiar to these individuals or whether other family members have similar characteristics.

The eight non-specific mental retardation conditions listed in Table 15 have localizations which appear to overlap the interval containing the MR locus in Family I. In five of the families in which MRX numbers have been assigned (MRX4, MRX5, MRX12, MRX13 and MRX17) hyperactivity and speech delay were part of the phenotype of the affected males in addition to mild to moderate mental retardation. In two of the families (assigned to loci MRX4 and MRX7), the affected males had delayed motor milestones as well as behavioural problems. Most of the affected males in these families were non-dysmorphic although some individuals were described as having an unusual facies. In the family assigned to MRX8 (Schwartz et al., 1992), the affected males were microcephalic. They also have a sloping forehead and upslanted palpebral fissures although the unaffected males also have these features (except with normal head circumferences). In the family reported by Schwartz et al. (1991) the mental retardation ranged from mild to severe, but in the other families listed in Table 15, the mental retardation was generally mild to moderate (as in Family I). The two-point linkage analyses in these reports usually resulted in LOD scores of just over +2 and in some cases the linkage analysis was carried out with penetrance set at 0.8 (in the linkage analysis carried out in Family I full penetrance was assumed).



The LOD scores obtained in Family I were well above +2 and the localisation of the disorder was narrowed to an interval of 10cM. A distance of 10cM would be of practical use if there were any females who required prenatal diagnosis or carrier detection but localisations spanning distances greater than this would probably be of little use in a diagnostic situation. It is not known whether any of the relatives of individual I:2 in Figure 35 are affected with the same condition. A female cousin of II:5 is a resident of a mental institution but this is obviously a different condition to the one segregating in the part of the family shown in Figure 35, otherwise it would imply that male to male transmission had occurred. Although the individual II:8 was reported to be "slightly dull intellectually" this was not felt on clinical grounds to be the same condition. This is supported by the observation that II:8 has inherited the opposite haplotype, for the candidate region, to the other affected males. In spite of this, I felt it necessary to see what effect recoding him as affected would have on the two-point linkage analysis. This resulted in negative LOD scores with markers which had previously given positive scores (Table 14), apart from *DXS566* and *DXS986* which both still gave positive LOD scores (of 1.67). Oddly, after this recoding, a negative LOD score of -2.91 was obtained with the *PGKI* locus which is located between these two loci. Inspection of the haplotypes in generation II showed that this is presumably due to the fact that the mother II:8 (I:2) is heterozygous at *PGKI* but homozygous at the flanking markers *DXS566* and *DXS986*. Therefore, changing the affection status of II:8 has a large effect on the family's two-point LOD score with *PGKI*, but little effect on the score with *DXS566* and *DXS986*.

The DNA studies in this family illustrate two important points concerning a linkage analysis of this type. First, it is crucial to have the correct clinical information for each individual because, as shown above, if this is not available or is ambiguous, it can have a dramatic effect on the outcome of the analysis. Secondly, it shows how the variability in polymorphism can make substantial differences to the LOD scores. The former may be a general problem with the MRX disorders. In some cases this may be due to a very mild phenotype, or it could be due to insufficient phenotype analysis. As pointed out by Schwartz (1993), the original Martin-Bell (fragile X

syndrome) family was originally reported as a family with a non-specific form of mental retardation. Conversely, the family described by Proops et al. (1983) which was reported as a Martin-Bell family, was later shown to be fragile X negative, with the MRX gene located in distal Xp (Arveiler et al., 1988a).

On the other hand, that linkage analysis results may dictate re-assessment of a clinical diagnosis is illustrated in the next chapter. A family with XLMR is described in which the mental retardation and associated spastic diplegia were originally thought to be due to X-linked hydrocephalus and MASA syndrome. Re-analysis of the pedigree using polymorphic DNA markers and re-evaluation of the clinical features of the affected males indicates that a new XLMR syndrome has been identified.

## 4. Localization of an X-linked mental retardation gene in Xp22.

### 4.1 INTRODUCTION

Unlike the non-specific X-linked mental retardation conditions (MRX), the XLMR syndromes (MRXS) usually have characteristic and well defined, though highly variable, phenotypes. Moderate to severe mental retardation is usually the only common feature. It is the presence of these distinctive phenotypes which facilitates mapping of XLMR syndromes; if the clinical features of several families are thought to represent the same syndrome, linkage data from more than one family can be combined (as has been possible in mapping of the Coffin-Lowry syndrome locus). Higher LOD scores are often obtained as a result. However, as described below, the presence of such a distinctive phenotype actually led to erroneous assumptions about the nature and location of an XLMR gene, in spite of early linkage data which suggested a gene location elsewhere on the X chromosome.

The family described in this chapter (Family J) was first reported more than 20 years ago (Fried, 1972) and at that time consisted of five mentally retarded males in one generation and a sixth affected male (deceased) in a previous generation. The degree of mental retardation varied from moderate to severe within the family and there were also two cases of hydrocephalus, one of which was reputedly due to aqueductal stenosis (this is described more fully in Chapter 5). All the affected males had delayed motor development and difficulty in walking as adults. At the time of this initial investigation DNA analysis was not possible, but a biochemical study by Fried and Sanger in 1973 using the polymorphic properties of the *Xg* blood group in Xp22.33 suggested possible linkage between this locus and the mental retardation gene segregating in the family. The gene which encodes the *Xg* blood group antigen has recently been identified as *PBDX* (pseudoautosomal boundary divided on the X chromosome; Ellis et al., 1994). Four out of five affected males who were analysed in Family J were *Xg*(a+) and five out of seven unaffected males were *Xg*(a-). These

results produced a maximum likelihood of linkage, between the XLMR gene and *Xg*, of 20.2 at  $\theta = 0.11$ . The family was not investigated again until 1989.

At this time, the clinical presentation of the affected males, of mental retardation, hydrocephalus and spastic diplegia was very suggestive of MASA syndrome which was first documented in 1974 (Bianchine and Lewis), later localized to Xq28 (Winter et al., 1989), and subsequently found to result from mutations in *LICAM* (Jouet et al., 1994). For these reasons, in spite of the initial suggestion by Fried that the gene responsible for the mental retardation and hydrocephalus in this family was probably linked to *Xg*, family J has been assumed by others (including Willems et al. (1990) and the London Dysmorphology Database) to be an example of the allelic MASA/HSAS syndromes (see Table 4) and by OMIM (Online Mendelian Inheritance in Man) to be a non-specific mental retardation syndrome (MRX1). It was important, therefore, to establish whether the gene responsible for this family's disorder was situated in Xp near the *Xg* locus, or in Xq28. Linkage to another region of the X chromosome was also possible.

Because of the similarities of the phenotype to the MASA syndrome phenotype, an extensive linkage analysis using DNA markers in Xq28, in the region of the *LICAM* locus was undertaken. When linkage to this region was excluded, other regions of the X chromosome were screened using markers at widely spaced intervals until there was evidence of linkage to a particular region, when the concentration of markers was increased. Most of the X chromosome was covered, extending from close to the pseudoautosomal boundary in Xp22.33 to distal Xq28, with variable coverage of markers across the chromosome.

## 4.2 FAMILY STUDIES

The pedigree of Family J is shown in Figure 39 (page 141). Each of the five affected males in generation III are sons of five sisters; the sixth affected male was their uncle (II:5). Three of the affected males are still alive in three different mental institutions in Scotland. In order to update the clinical information it was felt necessary to re-

examine the affected males in the family, although this was only possible on one individual (III:26) who was examined by Drs. David Bonthron and Alan Wright (University of Edinburgh, Human Genetics Unit and MRC Human Genetics Unit, respectively). New information was also obtained about the other two living affected males (III:35 and III:41) from the mental institutions in which they were resident.

#### **4.2.1 Case reports**

Case 1 (III:26) was born in 1947 after a normal delivery although his mother had been admitted to hospital with oedema one week prior to delivery. He weighed 8lbs at birth. He developed normally at first, but at 9 months suddenly regressed and developed loss of muscle tone with associated muscle wastage. Thereafter his development was slow; he walked at 3 years of age and talked at 4 years. He attended special schools from the age of 6 until he was 16 but was irritable and very difficult to manage at home. He was eventually admitted to a mental institution when he was 22 years old. His IQ was 39 (Binet). He had kyphosis with mild scoliosis and mild pes planus. His facial appearance was normal although at the time of his most recent examination in 1995, he was described as having coarse features (Figure 40, page 142). When he was 31 he was referred to a neurologist because he was unsteady on his feet and had difficulty walking; he was found to have weakness, sensory loss and impaired coordination in both legs with an extensor plantar reflex on the right side. He was diagnosed as having a progressive neurological disorder of unknown cause. One year later he was confined to a wheelchair. A skull X-ray carried out two years later showed bilateral symmetrical intracranial calcification affecting the basal ganglia. When he was examined again in 1995, he was noted to have increased tone in his arms and legs, lower limb weakness, bilateral extensor plantar reflexes and bilateral ankle clonus. A CT scan performed at this time showed extensive calcification of the basal ganglia and enlarged lateral ventricles (Figure 41, page 143).

Case 2 (III:35) was born in 1936 after a normal pregnancy and delivery. He had delayed developmental milestones and did not walk until 2 years of age or talk until



he was 2½ years old. He attended a special school from the age of eight and was admitted to a mental hospital when he was 45 with an IQ of approximately 45. His facial appearance was normal but he had mildly dysplastic ears. At the age of 47 he was referred to a neurologist because of weakness and confusional episodes. He was found to have bilaterally increased limb reflexes and some thinning of the interosseous muscles in the hands.

Case 3 (III:41) was born in 1951. Neurological examination at the age of 20 years showed an abnormal gait and spastic diplegia with exaggerated limb reflexes. Further examination at the age of 40 showed that he had kyphosis, mild choreic involuntary movements, a wide-based flat footed gait, and wasting of the small muscles of the hands. The plantar reflexes were extensor and abdominal reflexes were present. He had poor speech and an IQ of approximately 40. A skull X-ray in 1995 showed calcification of the basal ganglia (Figure 42, page 146).

The three cases described above all had a normal 46,XY karyotype and were negative for the cytogenetic fragile site (*FRAXA*).



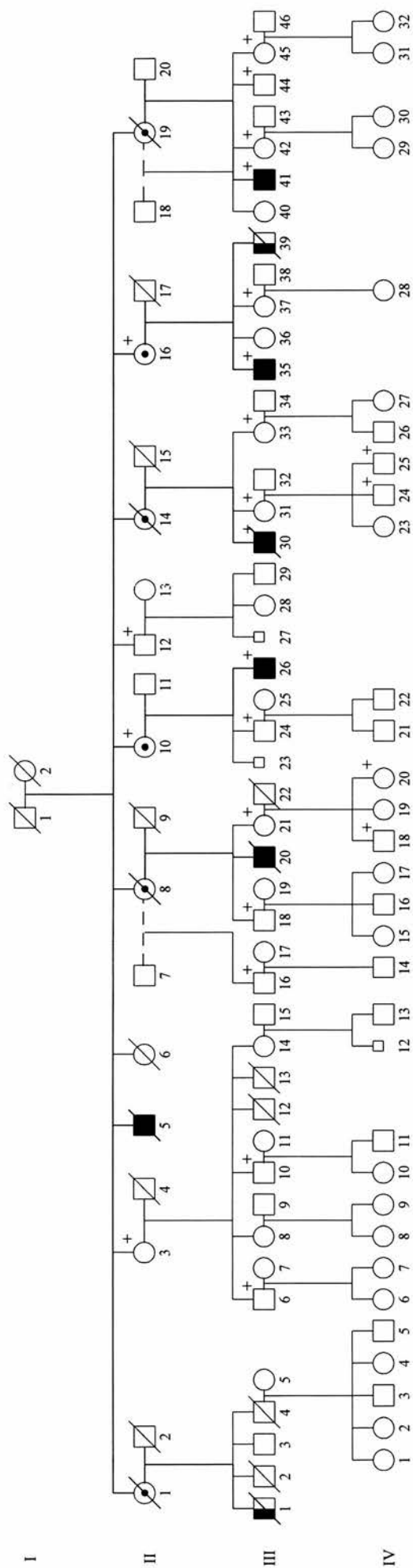


Figure 39. Pedigree of Family J. Affected males are denoted by filled black symbols, obligate carrier females are represented by symbols with a dot. The two males who were reported to have had hydrocephalus (III:1 and III:39) are shown by half shaded symbols. Blood was obtained from those individuals marked with a cross.



Figure 40. Individual III:26 at 47 years of age.

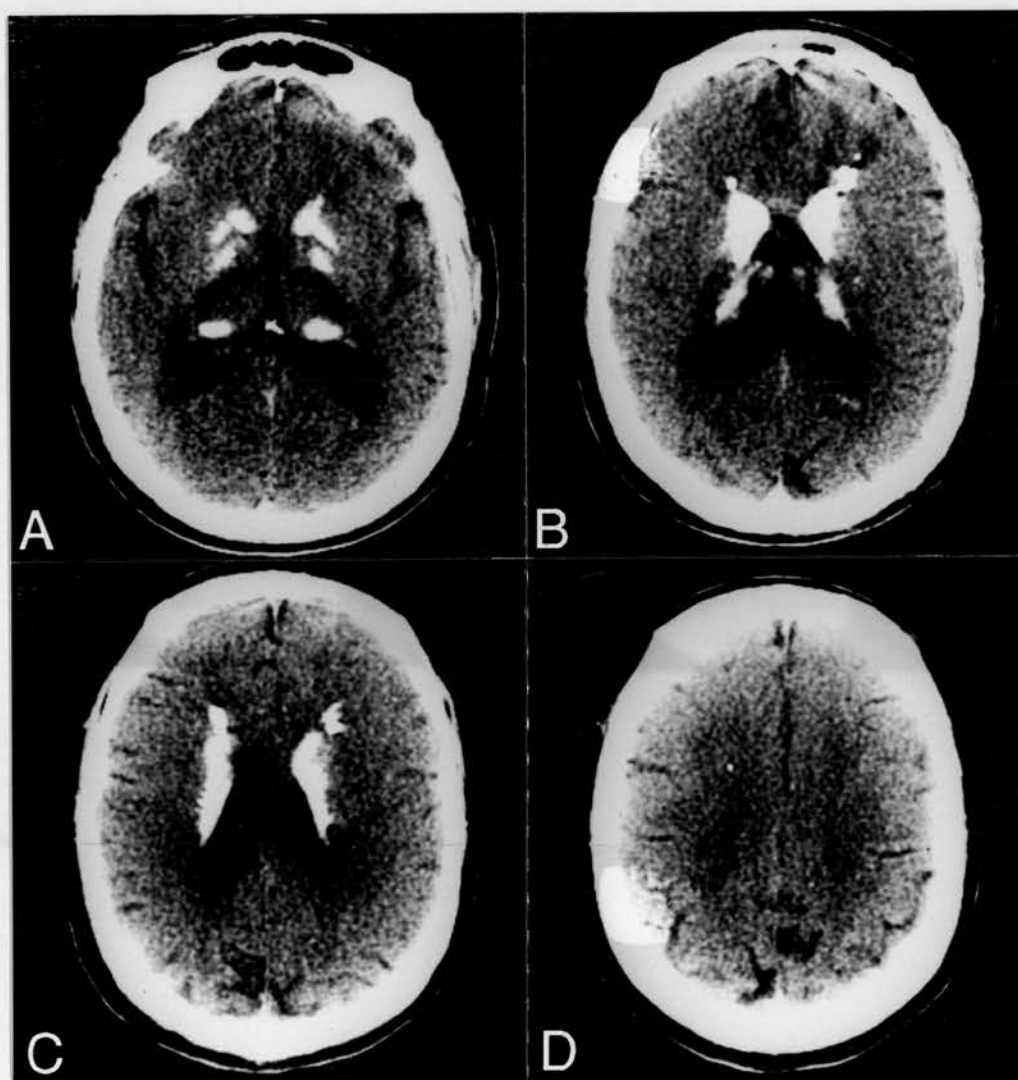


Figure 41. CT scan of individual III:26 showing bilateral calcification of the basal ganglia and lateral ventricular dilatation. Portions A to D are transverse sections of the skull at 1 cm intervals moving upwards.

There are also five deceased affected males in this family:

- 1) Individual II:5 was described as a severely handicapped male who never attended school and who had difficulty in walking. He was also described as having an unsteady gait, scoliosis and poor muscularity. He died in a mental hospital at age 40 from pulmonary tuberculosis. No post-mortem was carried out.
- 2) Individual III:1 was a severely handicapped male who was noted to have macrocephaly in infancy and never walked. He died at age 15 from meningitis although his death certificate stated "congenital hydrocephalus".
- 3) Individual III:20 was a mentally handicapped male with dorsolumbar scoliosis and wasting of calf muscles. Neurological examination in 1972 revealed cerebellar ataxia and spastic diplegia with exaggerated reflexes (plantar reflexes extensor and abdominal reflexes absent). He died when he was 33 years old from a suspected heart attack.
- 4) Individual III:30 was born in 1938 after a normal pregnancy although labour was prolonged and he was delivered by forceps. His milestones were delayed; he was unable to sit up until 8-9 months and did not walk unaided until he was 2½ years old. Although he attended school he was described as ineducable and never learned to read or write. He was admitted to a mental hospital on a permanent basis at age 16; at this time his IQ was 25. His legs were reported to be thin and he had bilateral genu valgum, pes planus and walked with a clumsy gait. He was also noted to have a posterior curvature of the upper third of his spine. No information is available about the cause of death.
- 5) Individual III:39 was born in 1955 with probable hydrocephalus. His head was noted to be large at the age of six weeks and at 6 months his OFC was 53cm (>97th percentile). Ventriculography showed gross symmetrical hydrocephalus with a small 4th ventricle and a suspected block in the aqueduct of Sylvius. He

underwent a ventriculo-peritoneal drainage operation at 6 months of age. He only spoke a few words, was able to stand with support, but could not walk unaided. He died when he was 4 years old.

The phenotype of the affected males in this family, therefore, is moderate to severe mental retardation in association with a progressive neurological disorder and spastic diplegia (which is, in fact, similar to the MASA phenotype). Wasting of the small muscles of the hand was also apparent in two of the three living affected males. The facial dysmorphism is very subtle; individual III:26 (Figure 40) has a long face with long ears and has coarse facial features; he has been described as having a facial appearance similar to males with fragile X syndrome. Although macrocephaly was present in infancy in the two males who were reported to have had hydrocephalus, the other mentally retarded males had normal head circumferences. Of the two males who had recent skull investigations (CT scan and X-ray) both had marked intracranial calcification of the basal ganglia, and one had enlarged lateral ventricles (it is unknown if III:41 also had enlarged ventricles because this could not be detected on an X-ray).

None of the obligate carrier females (II:8, II:10, II:14, II:16 and II:18) were known to have any manifestations of the disorder and all were of normal intelligence.

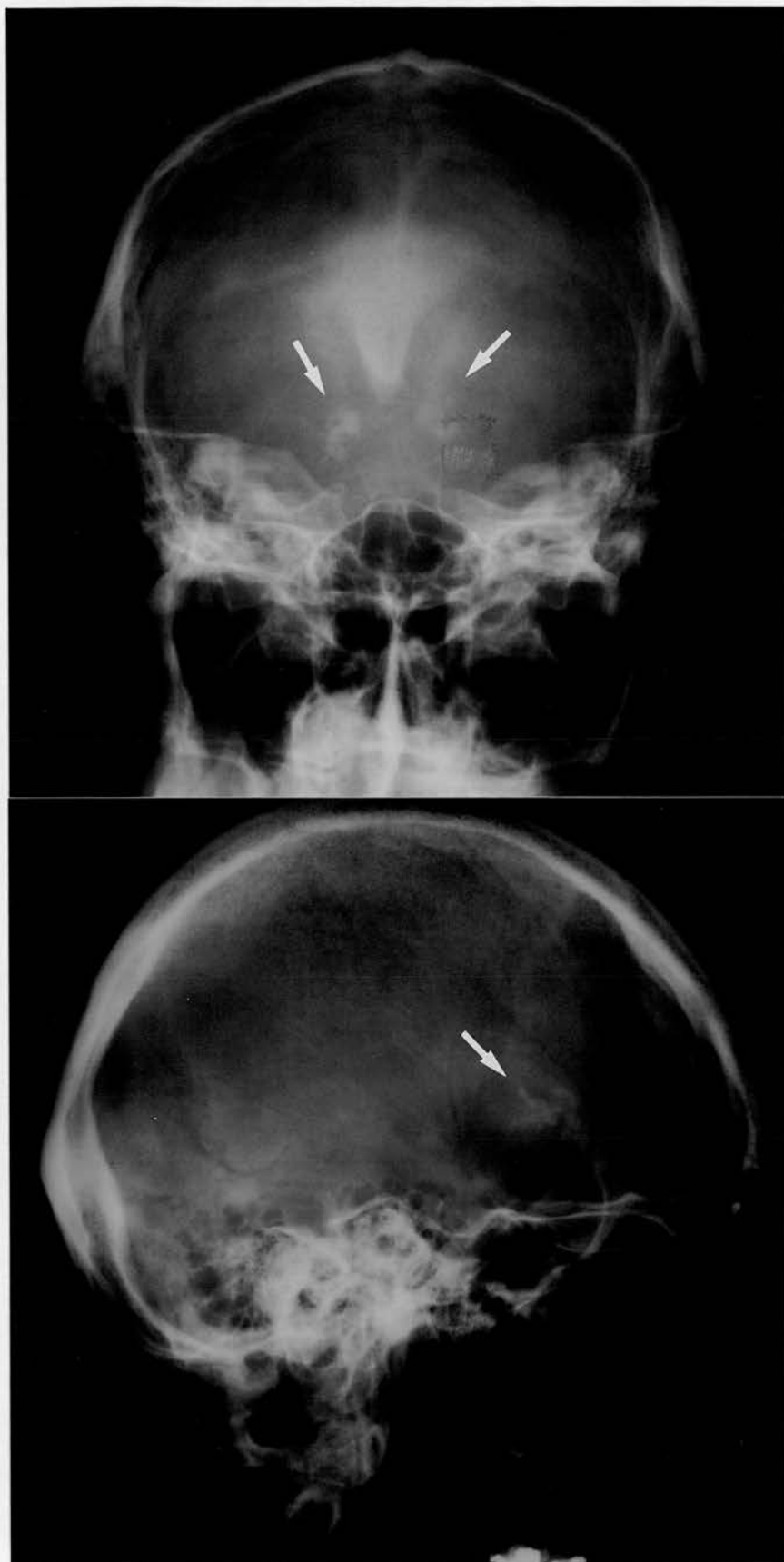


Figure 42. Skull X-rays of individual III:41 showing bilateral calcification of the basal ganglia.



### 4.3 METHODS

#### 4.3.1 DNA analysis

Blood or lymphoblastoid cell lines had been obtained from some family members in the 1980's and were stored in the MRC Human Genetics Unit, Edinburgh. DNA samples were transferred to our laboratory in 1989 and fresh blood samples were obtained from some family members. DNA was extracted from these new samples as described in 6.1.1.1. DNA was available on individuals marked on the pedigree (Figure 39, page 141) with a cross. As it was presumed that the syndrome involved in family J was MASA, the initial analysis concentrated on markers in distal Xq (the gene responsible for MASA had been localised to Xq28 in 1989; Winter et al.) and was carried out by Southern blotting and hybridization with radioactive probes, and also by PCR analysis. The details of these markers are shown in Tables 16 and 17.

Location	Locus	Probe	Enzyme	Alleles (kb)	Reference
Xq27.1	<i>DXS98</i>	4D-8	<i>MspI</i>	25, 7.8	Boggs and Nussbaum (1984)
Xq27.3	<i>DXS297</i>	VK23B	<i>XmnI</i>	10.3, 6.6	Suthers et al., (1991b)
Xq28	<i>DXS296</i>	VK21A	<i>TaqI</i>	10.9, 9.9	Suthers et al., (1989)
Xq28	<i>DXS305</i>	St35-691	<i>PstI</i>	5.1, 2.9/2.2	Vincent et al., (1989)
Xq28	<i>F8C</i>	F814	<i>BclI</i>	1.2, 0.9	Heilig et al., (1988)

Table 16. DNA markers in distal Xq analysed by Southern blotting and hybridization with radioactive probes.

Location	Locus	Reference	Primers (5'- 3')	Anneal temp.
Xq27.3	<i>FMRI</i>	Fu et al., (1991)	F GCTCAGCTCCGTTTCGGTTTCACITCCGGT R AGCCCCGCACTTCCACCACCAGCTCCTCCA	65°C
Xq28	<i>F8C</i> (int. 13 CA repeat)	Lalloz et al., (1991)	F TGCATCACTGTACATATGTATCTT R CCAAATTACATATGAATAAGCC	49°C
Xq28	<i>F8C</i> ( <i>HindIII</i> RFLP, int. 19)	Graham et al., (1990)	F GGCGAGCATCTACATGCTGGGATGAGC R GTCCAGAAGCCATTCCCAGGGAGTCT	60°C

Table 17. Distal Xq markers which were analysed by PCR.

When linkage to Xq28 was excluded (see LOD score results in Table 19, page 153) an extensive search for linkage to other regions was undertaken. Only DNA markers which could be analysed by PCR and detected using the A.L.F. sequencer were used, partly for ease of analysis, and also because DNA was limited on some family members. The details of the other markers which were analysed are shown in Table 18.

#### **4.3.2 Linkage analysis**

Two-point linkage analysis was carried out using MLINK. An arbitrary mutation rate of  $1 \times 10^{-5}$  and a corresponding gene frequency of  $3 \times 10^{-5}$  were used for the MLINK calculation. Deceased individuals II:5, III:20 and III:30 were coded as affected and complete penetrance for the disease was assumed. The alleles of each of the microsatellites were assumed to occur at equal frequencies and for the dimorphic markers, the published frequencies were used. The results of the pairwise analysis are shown in Table 19 (page 153).

Multipoint analysis was also carried out using the LINKMAP component of FASTLINK using genetic distances as shown in Figure 44 (page 152) by carrying out 15 overlapping five-point analyses (four marker loci and the disease locus). For the purposes of the LINKMAP analysis the mutation rates were set at zero. Genetic distances were converted into recombination fractions using the Kosambi mapping function prior to the calculation and reconverted to cM afterwards. Multipoint LOD scores were calculated from location scores which were obtained from the linkage report programme (LRP). These were plotted against genetic distance (using the marker *DXS996* as the starting point) and the final graph is shown in Figure 45 (page 154).

Location	Locus	Reference	Primers (5'- 3')	Anneal temp.
Xp22.3	<i>DXS996</i>	Gyapay et al., (1994)	F AAATTCTTGCCTAGGCCACTCTAGG R ACGTTGTTCTGGATCGTATGCTAGG	62°C
Xp22.32	<i>DXS237</i>	Gedeon et al., (1992b)	F CATGTGAGGAATACAGGAGCGAA R GCCAACTCAGCTAATGACTGTAG	55°C
Xp22.31	<i>KAL</i>	Bouloux et al., (1991)	F CCCAAAGTAAGGATTTTGCCAC R TAGATCCTATTTGCCAATTTTGG	55°C
Xp22.31	<i>DXS1224</i>	Gyapay et al., (1994)	F CTTCAAGCCTACAAAATCTGG R ACNTTAAAAGCCTGGTTCTTCTAAA	59°C
Xp22.2	<i>DXS987</i>	Gyapay et al., (1994)	F GTTGAGATAATGAGGCCAGT R ACNTTAAAAGCCTGGTTCTTCTAAA	52°C
Xp22.2	<i>DXS207</i>	Oudet et al., (1992)	F CTCTTGTCAGATTTTCAGGTCC R GGAATACTTGTAAGTTCATTGGGA	52°C
Xp22.2	<i>DXS1053</i>	Gyapay et al., (1994)	F TTAAGGAAGTATGAGGCTCCA R TTGGTGCAAAAAGTAATCACG	57°C
Xp22.1	<i>DXS418</i>	Van De Vosse et al., (1993)	F TGTGAGGTTTTGTTCCCTCC R GACTGTTGAGTTTCCTCACAGC	58°C
Xp22.13	<i>DXS999</i>	Gyapay et al., (1994)	F GCTAACAACCTAGACTTCAACC R CAGTTTCACAATCTCTGCC	50°C
Xp22.13	<i>DXS1229</i>	Gyapay et al., (1994)	F TAGAATCAACATAAGGCCCA R GGATACAATTAGCAGAATCAGACT	61°C
Xp22.13	<i>DXS1683</i>	Econs et al., (1994)	F GAGTTGTGAGAAAGAGCAGTA R AATGCCAGGTAACAACCTTTAAG	63°C
Xp22.13	<i>DXS1052</i>	Gyapay et al., (1994)	F GCATGTGTCAAGGCTGATT R TAACCTCTCTCGCTCGCTCT	61°C
Xp22.12	<i>DXS989</i>	Gyapay et al., (1994)	F ACATAAGAAATAAAATGGCTGC R AGAAGATAGATATACCCACTCACC	50°C
Xp21.2	<i>DXS1234</i>	Beggs & Kunkel (1990)	F GAAAGATTGTAAACTAAAGTGTGC R GGATGCAAAACAATGCGCTGCCTC	65°C
Xp21.1	<i>5' DYS-II</i>	Feener et al., (1991)	F TCTTGATATATAGGGATTATTTGTGTTTGTATA R ATTATGAAACTATAAGGAATAACTCATTTAGC	55°C
Xp11.23	<i>DXS426</i>	Coleman et al., (1990)	F CCTTCATCTCTACCAAGATA R CTGCACTCCAGCCTGAATAA	52°C
Xq12	<i>AR</i>	Sleddens et al., (1992)	F TCCGCGAAGTGATCCAGAAC R CTTGGGGAGAACCATCCTCA	55°C
Xq21.33	<i>DXS3</i>	Stanier et al., (1991)	F AATACATAGGTGTATTGTGACC R CCACCTCTCTGAAAGTGTGT	55°C

Table 18. DNA microsatellites covering the X chromosome (apart from Xq27 and q28) which were analysed by PCR. The marker *KAL* is a CA repeat at the Kallmann syndrome locus, *5' DYS-II* is one of two CA repeats at the 5' end of the dystrophin gene and *AR* is a trinucleotide repeat (CAG) at the androgen receptor locus

## 4.4 RESULTS

### 4.4.1 DNA analysis

A total of 33 X chromosome markers were analysed in family J and of these, 26 were informative, i.e. either the possible carrier II:3 or the obligate carriers II:10 and II:16 were heterozygous. The results of markers which gave positive LOD scores (Table 19) are shown on the pedigree in Figure 43. In the construction of this pedigree, the haplotypes were arbitrarily drawn as if individual II:3 had inherited a recombinant X chromosome from her mother and the other females in generation II are non-recombinants. The black bars represent the affected haplotype which was derived from the carrier I:2; she is assumed to be an obligate carrier because of the presence of II:5. If II:5 had not been affected it could have been possible that the father of the six carrier females in generation II was a gonadal mosaic for normal and mutant alleles and had transmitted the mutation to five out of six daughters. We can only assume that II:5 was affected with the same condition as the living affected males, from evidence in the report by Fried (1972).

Other markers which were analysed in family J but were not informative were: *DXS52* (St14), *DXS15* (DX13), *DXS105* (55.E), *DXS523* (pDK9.5), *DXS443*, *DXS365*, *DXS228* and *DXS1226*.

### 4.4.2 Linkage analysis

The LRP program was used to produce a table of two-point LOD scores (Table 19). Positive scores from *DXS1224* (Xp22.31) to *DXS1052* (Xp22.13) were obtained with a maximum LOD score of 4.22 at the *DXS418* locus (Xp22.1). However, two-point linkage analysis cannot define precisely the region of localization of the MR locus in this family which may be anywhere between *KAL* (Xp22.31) and *DXS989* (Xp22.12) and therefore LINKMAP analysis was carried out. The maximum multipoint LOD score obtained using markers from Xp22.3 to Xq28 (175cM) was 4.26 with the marker *DXS207* and the adjacent marker *DXS999* (Figure 45).



Xpter ↑	cM	$\theta$
<i>DXS996</i>		
	1.0	0.01
<i>DXS237</i>		
	3.3	0.033
<i>KAL</i>		
	7.6	0.075
<i>DXS1224</i>		
	3.7	0.037
( <i>DXS1053</i> )		
	0.0	
( <i>DXS987</i> )		
	0.0	
<i>DXS207</i>		
( <i>DXS418</i> )		
	2.6	0.026
<i>DXS999</i>		
	4.8	0.048
<i>DXS1229</i>		
	8.0	0.079
( <i>DXS1052</i> )		
( <i>DXS1683</i> )		
<i>DXS989</i>		
	7.4	0.073
<i>DXS1234</i>		
	8.3	0.082
<i>5'DYS</i>		
	22.5	0.211
<i>DXS426</i>		
	6.5	0.065
<i>AR</i>		
	15.4	0.149
<i>DXS3</i>		
	56.9	0.407
<i>DXS98</i>		
	12.5	0.122
<i>DXS297</i>		
	3.5	0.035
<i>FMR1</i>		
	0.6	0.006
<i>DXS296</i>		
( <i>DXS305</i> )		
	11.6	0.114
<i>F8C (F814)</i>		
	0.0	
<i>F8C (int.13)</i>		
	0.0	
<i>F8C (int.19)</i>		
↓		
Xqter		

Figure 44. Genetic distances between informative markers analysed in Family J. Figures on the left are in cM and were taken from Fain et al., (1995). For the LINKMAP analysis these genetic distances were converted from cM to recombination fractions (shown on the right) using the Kosambi mapping function. Markers in brackets were excluded from the LINKMAP analysis because, either the genetic distances involving these markers were unobtainable, or the distances between two adjacent markers was zero. Of the three markers at the *F8C* locus, the CA repeat in intron 13 was used in the LINKMAP analysis (because it is more polymorphic).



LOD scores at $\theta$ of:									
Locus	0.0	0.01	0.05	0.1	0.2	0.3	0.4	$Z_{\max}$	$\theta_{\max}$
<i>DXS996</i>	$-\infty$	1.85	2.29	2.25	1.83	1.24	0.57	2.30	0.06
<i>DXS237</i>	$-\infty$	1.85	2.29	2.25	1.83	1.24	0.57	2.30	0.06
<i>KAL</i>	$-\infty$	1.97	2.39	2.33	1.88	1.25	0.54	2.40	0.06
<i>DXS1224</i>	2.31	2.28	2.16	1.99	1.62	1.17	0.63	2.31	0.00
<i>DXS987</i>	4.04	3.99	3.76	3.46	2.75	1.91	0.95	4.04	0.00
<i>DXS207</i>	3.26	3.21	3.00	2.71	2.10	1.42	0.68	3.26	0.00
<i>DXS1053</i>	1.69	1.66	1.53	1.36	1.00	0.62	0.23	1.69	0.00
<i>DXS418</i>	4.22	4.15	3.89	3.54	2.78	1.91	0.93	4.22	0.00
<i>DXS999</i>	1.83	1.80	1.67	1.50	1.12	0.71	0.28	1.83	0.00
<i>DXS1229</i>	1.77	1.73	1.59	1.41	1.04	0.65	0.25	1.77	0.00
<i>DXS1683</i>	2.36	2.33	2.21	2.05	1.67	1.21	0.66	2.36	0.00
<i>DXS1052</i>	3.56	3.51	3.27	2.97	2.30	1.56	0.75	3.56	0.00
<i>DXS989</i>	$-\infty$	2.45	2.89	2.84	2.38	1.69	0.83	2.90	0.06
<i>DXS1234</i>	$-\infty$	0.46	1.60	1.88	1.76	1.31	0.67	1.90	0.12
<i>5' DYS</i>	$-\infty$	-3.83	-1.21	-0.25	0.41	0.50	0.32	0.50	0.27
<i>DXS426</i>	$-\infty$	-4.98	-2.40	-1.47	-0.80	-0.51	-0.24	0.00	0.50
<i>AR</i>	$-\infty$	-4.65	-2.00	-0.99	-0.19	0.07	0.11	0.11	0.37
<i>DXS3</i>	$-\infty$	-5.19	-2.51	-1.45	-0.55	-0.18	-0.03	0.00	0.50
<i>DXS98</i>	$-\infty$	-6.71	-3.32	-1.95	-0.79	-0.31	-0.11	0.00	0.50
<i>DXS297</i>	$-\infty$	-3.03	-1.61	-1.02	-0.52	-0.29	-0.13	0.00	0.50
<i>FMR1</i>	$-\infty$	-10.70	-5.87	-3.85	-1.96	-0.99	-0.40	0.00	0.50
<i>DXS296</i>	$-\infty$	-3.57	-1.71	-1.10	-0.78	-0.64	-0.31	0.00	0.50
<i>DXS305</i>	$-\infty$	-2.89	-1.54	-1.04	-0.73	-0.59	-0.32	0.00	0.50
<i>F8C (F814)</i>	$-\infty$	-3.30	-1.42	-0.79	-0.43	-0.42	-0.32	0.00	0.50
<i>F8C (int.13)</i>	$-\infty$	-13.46	-7.30	-4.75	-2.39	-1.17	-0.44	0.00	0.50
<i>F8C (int. 19 HindIII RFLP)</i>	$-\infty$	-3.30	-1.42	-0.79	-0.43	-0.42	-0.31	0.00	0.50

Table 19. Results of two-point linkage analysis.

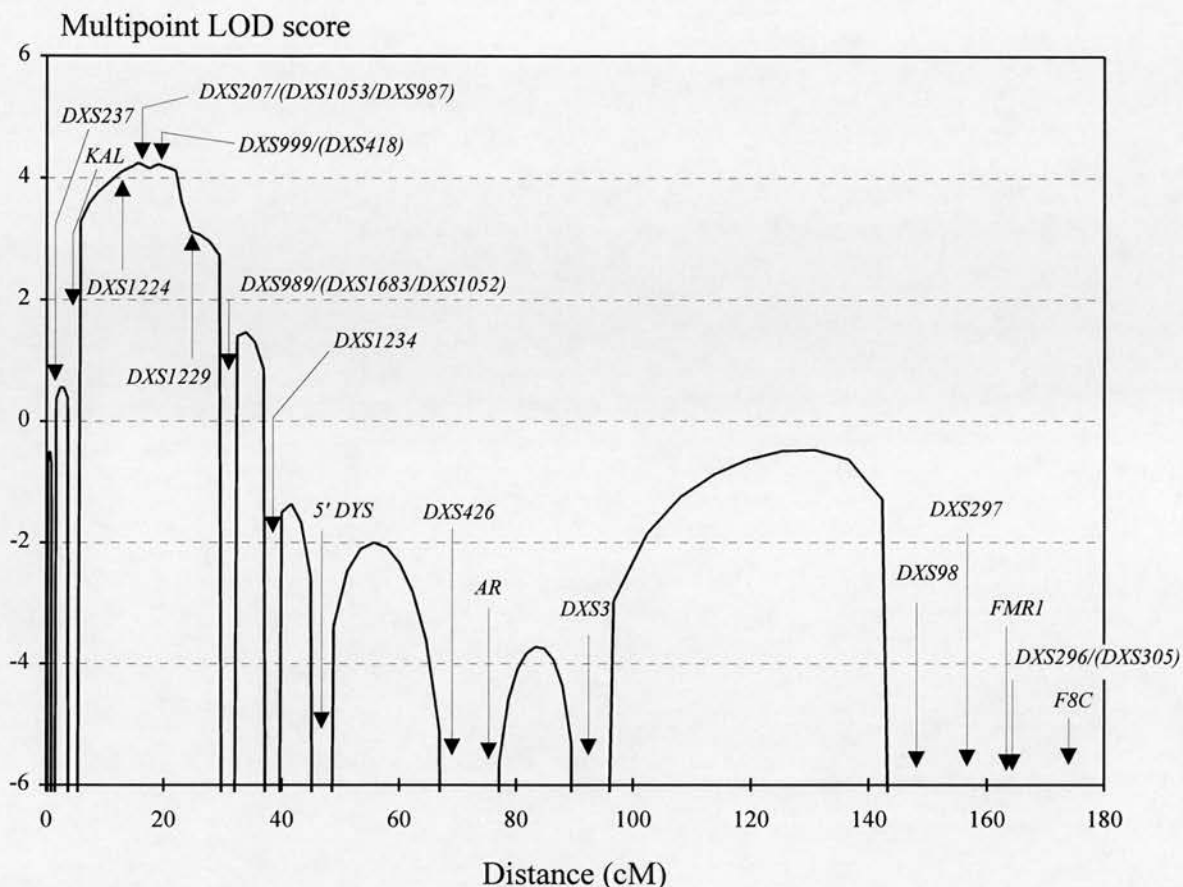


Figure 45. Results of LINKMAP analysis on microsatellite data on Family J using genetic distances as shown in Figure 44. The marker *DXS996* (not shown) was used as the starting point (0cM). The location scores produced by LRP were converted to multipoint LOD scores by dividing by  $2\ln(10)$  as described in Terwilliger and Ott (1994). A negative LOD score cut-off point of -6 was arbitrarily chosen. The method used in this LINKMAP analysis is that recommended by Terwilliger and Ott (1994) although LOD scores produced by this “sliding” method are not strictly comparable to a complete 18-point multipoint analysis. The maximum multipoint LOD score obtained in this analysis was 4.26 with the marker *DXS207*. Markers shown in brackets were excluded from the LINKMAP analysis for the reasons described in Figure 44. The shoulder between the markers *DXS999* and *DXS1229* may be a consequence of combining information from two 5-point analyses. Alternatively, it may reflect differences in informativeness of markers in this region.

## 4.5 DISCUSSION

Family J originally presented with moderate to severe mental retardation and spastic diplegia in 1972. However, more than 20 years later, the cause of their mental retardation has not been determined. Although Fried (1972) suggested that the MR locus in this family lay in the distal short arm of the X chromosome, and data presented here confirm this, the striking phenotype in this family led to the belief that the affected males had MASA syndrome. In addition, the presence of two males who were suspected of having hydrocephalus, and the recent discovery that one form of X-linked spastic paraplegia (SPG1) is also caused by *LICAM* mutations (Jouet et al., 1994), added weight to the assumption that the MASA/HSAS/SPG1 syndromes were involved. Therefore, early DNA analysis in family J (both by myself and by Dr Alan Wright, MRC Human Genetics Unit) concentrated on markers in distal Xq.

### Linkage analysis

When linkage to distal Xq was excluded, markers spanning the whole of the X chromosome were then analysed. The two-point linkage analysis resulted in a maximum LOD score at 4.22 with the marker *DXS418*. Positive LOD scores were also obtained with the markers surrounding *DXS418* although the absolute values vary as a reflection of the differences in informativeness of the markers. The important observation is the presence of several adjacent strongly positive LOD scores in one region and negative LOD scores elsewhere on the X chromosome. Two-point linkage analysis indicated, therefore, that the locus for the mental retardation gene segregating in Family J lies in Xp22.1-Xp22.3 between *DXS989* proximally and *KAL* distally; a distance of 26.7 cM (see Figure 43). Multipoint linkage analysis gave a maximum LOD score of 4.26 with the marker cluster *DXS207/DXS1053/DXS987* and the neighbouring cluster, *DXS999/DXS418*. If a confidence level of 2 units of LOD score is used (likelihood ratio of 100:1), the region containing markers with LOD scores above 2.26 are encompassed within the interval *KAL-DXS989*. Using a less stringent 1 LOD unit of support, the interval can be confined to that between the markers *DXS1224* and *DXS1229* which is a distance

of 17.2cM. Therefore, depending upon the level of confidence used, the interval containing the MR locus in Family J is either 17.2cM or 26.7cM.

Two lines of evidence, therefore, excluded MASA/HSAS as being implicated in the condition affecting Family J:

- a) Negative LOD scores were obtained with Xq28 markers and positive LOD scores obtained with distal Xp markers.
- b) Further evaluation of the phenotype showed several features which are absent in MASA but present in this family and vice versa.

Recent skull examinations in two of the affected males showed that they both had marked intracranial calcification of the basal ganglia (it is presumed to be calcium because of the density of the substance on X-ray). This is a feature which has not been seen in the MASA/HSAS syndromes although enlarged ventricles (as seen in individual III:26) have been observed in some MASA families (Boyd et al., 1993). Other features seen in this family include exaggerated limb reflexes, kyphosis, scoliosis, and wasting of the small muscles in the hands; the first two features have been seen in MASA patients, the latter have not. In addition, the males in family J did not have aphasia, although their speech was poor. None of the affected males had adducted thumbs.

Linkage data and new clinical evidence, therefore, have shown that the disorder segregating in family J is not one of the allelic MASA/HSAS/SPG1 syndromes, but a mental retardation/spastic diplegia syndrome with a gene localization in distal Xp. We can speculate whether this is the same as, or allelic to, other mental retardation syndromes in this region or whether the condition in family J represents a new XLMR syndrome.

### Other syndromes associated with mental retardation in Xp22.1-Xp22.3

- 1) Coffin-Lowry syndrome. This was first described Coffin et al. (1966), later by Lowry et al., (1971) and finally demonstrated to be a single entity by Temtamy et al. (1975). It is an X-linked semi-dominant condition characterized by severe mental retardation, speech delay, coarse facies with epicanthic folds, hypertelorism, thick lips and anteverted nostrils, large soft hands with puffy tapering fingers, hypotonia with hyperlaxity of joints, and various progressive skeletal abnormalities including pectus carinatum (pigeon chest), kyphosis (hump-back) and scoliosis (lateral curvature of the spine). Arrested hydrocephalus may also be a feature. Miyazaki et al., (1990) described a 22 year old man with Coffin-Lowry syndrome (CLS) whose cervical spinal canal was compressed by calcified ligamenta flava (fibrous ligaments between the vertebrae). He had a spastic gait and exaggerated reflexes in his legs. Biochemical analysis suggested that an alteration in glycosaminoglycan metabolism was a pathogenetic factor in calcification of ligamenta flava. Ishida et al., (1992) described 3 males with CLS who had calcification of the ligamenta flava as a result of calcium pyrophosphate dihydrate deposition disease which was interpreted as further evidence that a metabolic abnormality in collagen and proteoglycans is present in CLS. Linkage data presented by Biancalana et al., (1992) on 16 families using 7 RFLP markers spanning the Xp22.2-p22.1 region, placed the CLS locus, with a maximum multipoint LOD score of 7.3, within a 7cM interval defined by the *DXS207-DXS43-DXS197* cluster distally and *DXS274* proximally. Biancalana (1994), later refined the genetic localisation of the CLS gene to a 5 cM interval between *DXS7161* and *DXS1052* (the proximal limit is now defined by the marker *DXS1683* (Willard et al., 1994) by construction of a high-resolution linkage map. To date, no linkage heterogeneity has been detected.
- 2) Snyder-Robinson syndrome. In 1969 Snyder and Robinson described a family with nine males with mental retardation over three generations. The males had hypotonia and dysequilibrium but no specific facial characteristics and the authors



concluded that their family had a non-specific form of XLMR. This family was restudied by Arena et al., (1994). DNA studies were conducted on 17 family members: 6 affected males, 3 carrier females, and 8 normal males and produced a maximum LOD score with *DXS41* in Xp22.13. The clinical features were: mild to moderate mental retardation, large head, asthenic body build (marfanoid habitus), diminished muscle bulk, nasal voice, high, narrow or cleft palate, long, thin asymmetrical face, mild joint hypermobility, long, thin fingers and great toes and mild to severe scoliosis. The phenotype suggests that the condition affecting this family is a syndromic disorder and not a non-specific XLMR.

- 3) Partington X-linked mental retardation syndrome (MRXS1). In 1988, Partington et al. described a family in which 10 males had mild to moderate mental retardation, recurrent dystonic spasms of the hands, and dysarthria. DNA linkage analysis gave a maximum LOD score with the marker *DXS41* (Xp22.2-Xp22.1) and analysis with flanking markers placed the locus for the MRXS1 syndrome in Xpter - Xp21.

Of the three syndromes described above, the one which most closely resembles the disorder in Family J is Coffin-Lowry syndrome, but only in terms of the mental retardation, ventricular dilatation and skeletal abnormalities. The other features of CLS are not consistent with those seen in Family J and the affected males have none of the facial features which are so characteristic of CLS. The most interesting observation is the deposition of calcium in the spinal canal although there is no evidence of intracranial calcification in CLS patients. It is conceivable that the disorder in Family J and CLS are allelic variants although this cannot be determined until the CLS gene has been identified.

#### Further investigations in this family?

DNA analysis in this family has confined the region of localization to between 17cM and 27cM (depending upon the level of confidence). This is a large distance in genetic terms (~9-14% of the X chromosome). This interval could probably be



narrowed considerably if microsatellites between *KAL* and *DXS1224* (7.6cM) and *DXS989* and *DXS1052* (0-8cM) were analysed; the actual interval of localization could be then anywhere from 11.1cM to 26.7cM.

According to the haplotypes shown on the pedigree in Figure 44, there are no women in generation III who have the affected haplotype and therefore no-one in generation IV is likely to require carrier testing or prenatal diagnosis in the future. Individuals III:21 and III:31 have recently enquired about their carrier status on behalf of their daughters (IV:19, IV:20 and IV:23) and both women were reassured that, as they did not have the same Xp22 haplotypes as the affected males, they were unlikely to be carriers of the disorder affecting their brothers. However, there is a remote possibility that a double recombination event has occurred in this region; two of the intervals between markers analysed in Family J are about 8cM which means that the chance of a double cross-over occurring but not being detected is  $6.4 \times 10^{-3}$ .

In this chapter, the distinctive phenotype of the affected males in Family J, that of mental retardation and spastic diplegia, misled others into believing that the condition was MASA/HSAS. The following chapter describes a family which presented with the classical HSAS phenotype (which was confirmed pathologically) and which was also found not to be linked to Xq28 markers.

## **5. Gonadal and somatic mosaicism mimicking genetic heterogeneity in X-linked hydrocephalus (HSAS).**

### **5.1 INTRODUCTION**

As illustrated in the previous chapter, linkage analysis was invaluable in demonstrating that the syndrome affecting Family J was not MASA/HSAS but an XLMR syndrome in the distal short arm of the X chromosome. Exclusion of MASA/HSAS was also verified by new clinical evidence. Two males in Family J died in childhood as a result of congenital hydrocephalus and in one of the males, this was reported to be due to aqueductal stenosis, which, in an X-linked pedigree is usually indicative of HSAS.

In this chapter, Family K is presented in which four males were unambiguously affected with aqueductal stenosis (HSAS) and in one case this was verified by neuropathological investigation. In contrast to the linkage studies carried out in the previous two chapters, linkage analysis in Family K confounded investigations by giving an impression of genetic heterogeneity in HSAS with a second locus near *FMRI*. This suggestion had to be reconsidered later, when the proband was shown to have an *LICAM* mutation which segregated with the disease in the family. The most likely explanation for the discordant linkage data is that the maternal grandmother of the affected males is a gonadal and somatic mosaic for the *LICAM* mutation.

#### **5.1.1 Congenital hydrocephalus**

Hydrocephalus is defined as an increase of free cerebrospinal fluid (CSF) in the ventricular system of the brain. Fluid formed in the lateral ventricles flows through the foramina of Monro into the third ventricle and from there through the Sylvian aqueduct into the fourth ventricle. CSF leaves the fourth ventricle and moves into the subarachnoid space at the back of the brain, from where it is gradually reabsorbed into veins or through the capillaries of the subarachnoid spaces.

Congenital hydrocephalus is a symptom of heterogeneous disorders rather than a specific diagnosis and is often associated with other central nervous system abnormalities. The majority of cases have no clear-cut etiology and could be due to a combination of genetic and environmental factors (Stoll et al., 1992). Most cases of congenital hydrocephalus are sporadic but a minority are inherited as either autosomal recessive (McKusick 236670 and 220200) or X-linked traits (McKusick 307000). The overall incidence of obstructive hydrocephalus is between 0.4 and 0.8 per 1000 live and stillbirths (Halliday et al., 1986).

### **5.1.2 X-linked hydrocephalus (HSAS)**

Sixty percent of cases of congenital hydrocephalus are male. This excess is due to the existence of the well defined X-linked recessive form (HSAS; hydrocephalus due to stenosis of the aqueduct of Sylvius). HSAS accounts for 7-15% of congenital hydrocephalus in males with a birth prevalence estimated at 1 in 30,000 (Halliday et al., 1986). The true prevalence may be higher than this because HSAS can present with atypical features and even without hydrocephalus [Willems et al., (1987); Serville et al., (1992)].

#### Background

Hereditary aqueductal stenosis was first reported by Bickers and Adams in 1949 and since then more than 40 families have been reported with the condition [Edwards (1961); Shannon and Nadler (1968); Holmes et al., (1973); Faivre et al., (1976); Cassie and Boon (1977); Søvik et al., (1977); Habib (1979); Landrieu et al., (1979); Renier et al., (1982); Van Egmond-Linden et al., (1983); Holden et al., (1990); Orth et al., (1991); Willems et al., (1987,1990,1992); Lyonnet et al., (1992); Serville et al., (1992); Jouet et al., (1993a,b, 1995); Ruiz et al., (1995)].

#### Pathogenesis

In HSAS, the hydrocephalus is due to narrowing or occlusion of the Sylvian aqueduct connecting the third and fourth ventricles which prevents fluid draining into the subarachnoid space from where it can be re-absorbed. This may be the result of

stenosis of the lumen, forking of the aqueduct, or complete or partial occlusion by a septum. As CSF formation is relatively constant, it is the change in resistance to absorption that determines whether the hydrocephalus is progressive. A normal aqueduct can permit moderate variations in the rate of production of CSF without an increase in third ventricular pressure but a stenotic aqueduct cannot. The increase in CSF volume leads to a rise in intracranial pressure; occasionally, the fetal head is so distended that perforation of the skull is necessary during delivery (Cassie and Boon, 1977).

### Clinical features of HSAS

HSAS is characterised by hydrocephalus, macrocephaly, flexion-adduction deformities of the thumbs (which are usually adducted) and forefingers, neurological abnormalities, such as spasticity of the lower limbs secondary to hypoplasia or absence of pyramidal tracts, enlarged ventricles and varying degrees of mental and physical handicap. Other cerebral malformations including agenesis of the corpus callosum and septum pellucidum have also been reported but all features are highly variable. Landrieu et al., (1979) postulated that aqueductal stenosis develops secondary to communicating hydrocephalus and may even be absent (Van Egmond-Linden, 1983). It has also been postulated that adducted thumbs are not secondary to a neurological defect but are defects in the development of the abductor and extensor muscles (Holtzman et al., 1976). The diagnosis of HSAS is not always easy; in the absence of a conclusive family history the arguments in favour are: the detection of hydrocephalus with aqueductal stenosis, one or both thumbs adducted, or the congenital absence of pyramids in sections of the medulla. Congenital absence of pyramids is observed in almost all cases of HSAS but is not pathognomonic.

### Prognosis and Treatment

The prognosis for males with HSAS is poor; the most severe cases die prenatally or shortly after birth with gross hydrocephalus and associated severe abnormalities. Attempts to arrest the hydrocephalus usually involve CSF shunting, via a ventriculo-peritoneal shunt. The poor developmental outcome in surgically treated patients

suggests, however, that the mental retardation is not secondary to the hydrocephalus but caused by associated brain abnormalities.

### 5.1.3 Localisation of HSAS to Xq28

In 1989, the HSAS gene was assigned to distal Xq28 close to Factor VIII (*F8C*) and *DXS52* by two-point linkage analysis in a family with 5 affected males (Willems et al.). Extensive linkage analysis of this family plus an additional three HSAS families was later carried out using 10 DNA markers spanning Xq26-Xq28 (Willems et al., 1990). A total of 6 affected males, 16 obligate heterozygotes and 14 unaffected males were analysed in this study. No recombinants between HSAS and *F8C* or *DXS15* were found in 20 and 15 informative meioses respectively; however, one recombinant between HSAS and *DXS52* in 30 informative meioses, placed HSAS and *DXS15* on the same side of *DXS52*. The relative order of the *DXS52*, *DXS15*, *F8C* cluster at this time was unknown but is now established as:

(*DXS52*, *DXS15*, **HSAS**, *F8C* → Xqter).

In 1992, Willems et al. extended their linkage study to analyse 21 probes from 18 Xq27-Xq28 loci in 13 HSAS families, including the four families from their previous study. Multipoint linkage analysis and key recombination events in these families indicated that the putative HSAS gene was situated between *DXS52* and *F8C*, a distance of 1-2 Mb by physical mapping data (Poustka et al., 1991). This localization was subsequently confirmed in additional families (Holden et al., 1990, Orth et al., 1991; Serville et al., 1992; Lyonnet et al., 1992) to be a 1.5 Mb region between *DXS52* and *DXS605* (Jouet et al., 1993a), by further linkage analysis. With the exception of one family (family 12) studied by Willems et al. (1992) there is currently no evidence for linkage heterogeneity although the authors pointed out that the clinical diagnosis in an unaffected individual in this family, who carried the affected haplotype, may have been incorrect.



#### **5.1.4 The *LICAM* gene and the *L1* cell adhesion molecule**

The only known gene in Xq28 in the 1.5Mb interval between *DXS605* and *DXS52* which had a function in neurological tissue was *LICAM* (Djabali et al., 1990). This encodes a neural cell adhesion molecule and was therefore considered as a candidate gene for HSAS [Willems et al., (1992); Rosenthal et al., (1992)]. The L1 molecule is a member of the immunoglobulin superfamily of neural adhesion molecules and is a highly conserved integral membrane glycoprotein involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites, cerebellar granule cell migration and interactions between epithelial cells of intestinal crypts (Moos et al., 1988). The  $\text{Ca}^{2+}$  independent neural adhesion molecules are important in the specification of cell interactions during development, maintenance and regeneration of the nervous system. Hlavin and Lemmon (1991) cloned and sequenced cDNA from human brain L1 and demonstrated that L1 supports neurite growth *in vitro*. The protein has a multidomain structure comprising six extracellular immunoglobulin domains of type C2 and five fibronectin type III domains. In human *LICAM*, the second Ig domain C2 (Ig2) and the second fibronectin type III (Fn2) show a high degree of conservation with the mouse (*Licam*), rat (*NILE*) and chicken (*Ng-CAM*) homologues and are therefore likely to be functionally important (Hlavin and Lemmon, 1991). In addition, the cytoplasmic domain shows interspecies conservation indicating that this portion of the molecule may also be involved in regulating cell adhesion.

#### **5.1.5 HSAS is caused by mutations in the *LICAM* gene.**

In 1992, Rosenthal et al. found a point mutation at a potential branch point signal in intron 18 of the *LICAM* gene which segregated with hydrocephalus in an HSAS family. Translation of the aberrantly spliced mRNA resulted in a protein with significant changes in the cytoplasmic domain. This was the first definite indication that *L1* was the gene involved in HSAS. Van Camp et al., (1993) subsequently screened 25 HSAS families for this point mutation but found it to be absent, although they did identify a 1.3 kb duplication which cosegregated with HSAS in one family.



To date, 20 mutations in the *LICAM* gene have been reported in HSAS families. These are listed in Table 20.

Author	Type of mutation	Position	Amino acid/nucleotide change
Rosenthal et al., 1992	A/C substitution	intron 18	Insertion/deletion
Van Camp et al., 1993	1.3kb duplication	exon 28	
Jouet et al., 1993b	G/A substitution	exon 7	Cys264Tyr
Coucke et al., 1994	5' splice site mutation	intron 4	
Jouet et al., 1994	G/A substitution	exon 6	Arg184Gln*
Jouet et al., 1994	G/A substitution	exon 11	Gly452Arg
Jouet et al., 1994	C/T substitution	exon 12	Arg485STOP
Jouet and Kenwrick 1995	G/T substitution	intron 1 donor splice site	
Jouet et al., 1995	G/C substitution	exon 1	Trp9Ser
Jouet et al., 1995	G/A substitution	exon 4	Gly121Ser
Jouet et al., 1995	A/T substitution	intron 10 splice junction	skipping of exon 10: deletion of 48 a.a.
Jouet et al., 1995	C/T substitution	exon 14	Gln586STOP
Jouet et al., 1995	G/T substitution	exon 18	Val768Phe
Jouet et al., 1995	G/T substitution	exon 21	Pro941Leu
Jouet et al., 1995	A/T substitution	exon 24	Tyr1070Cys
Gu et al., 1996	1bp insertion (C)	exon 1	Frameshift/9 novel a.a./STOP
Gu et al., 1996	A/G substitution	exon 6	Tyr194Cys
Gu et al., 1996	C/T substitution	exon 7	Pro240Leu
Gu et al., 1996	G/A substitution	exon 8	Trp276STOP
Gu et al., 1996	1 bp deletion (G)	exon 8	Frameshift/70 novel a.a./STOP

Table 20. Mutations found in *LICAM* in HSAS families. The Arg184Gln\* is the mutation found in the original HSAS family described by Bickers and Adams in 1949 and further characterized by Edwards et al. in 1961.

The *LICAM* gene consists of 28 exons spanning 15 kb. Nearly every form of mutation has been described so far (with the exception of whole gene deletions) with missense mutations being the most common, comprising more than 50% of all mutations. The mutations are distributed across the *LICAM* gene. It appears that the mutations which would predict truncation of the protein before the transmembrane domain and hence result in absence of *LICAM* function are associated with severe congenital hydrocephalus and early mortality (Kenwrick et al., 1996). Conversely, the mutations which appear in MASA patients result in a protein which is truncated in the cytoplasmic portion and presumably is expressed on the cell surface.

#### **5.1.6 MASA and SPG1 are also caused by mutations in the *LICAM* gene**

Two other related neurological disorders, MASA syndrome (mental retardation, aphasia, shuffling gait and adducted thumbs) and spastic paraplegia type 1 (SPG1) have also been shown to be caused by heterogeneous mutations in *LICAM* [Rosenthal et al., (1992); Jouet et al., (1993a, 1994); Jouet and Kenwrick, 1995)]. MASA syndrome was first reported in 1974 (Bianchine and Lewis) in a large Mexican/American family and since then at least 13 other families with more than 50 patients have been documented [Gareis and Mason,(1984); Yeatman,(1984); Winter et al., (1989); Schrandt-Stumpel et al., (1990); Rietschel et al., (1991); Straussberg et al., (1991); Macias et al., (1992); Legius et al., (1994)]. The main clinical features are summarised by the acronym, although there is considerable intrafamilial and interfamilial variability with mental retardation being the only consistent feature. Other findings include strabismus, ptosis, and musculoskeletal abnormalities including kyphoscoliosis, lordosis and torticollis (Boyd et al., 1993). Some of the features of MASA syndrome (which has also been referred to as clasped thumb mental retardation by some authors) are shared with X-linked spastic paraplegia type 1 (SPG1). In addition, although MASA and SPG1 patients do not generally present with hydrocephalus, enlarged lateral ventricles and agenesis of the corpus callosum have been reported in some MASA patients [Schrandt-Stumpel et al. (1992); Boyd et al. (1993)].

To date, more than 30 *LICAM* mutations have been reported in the MASA, HSAS and SPG1 conditions and it is apparent that each family has a different mutation and that the mutations are distributed across the gene i.e. there are no common mutations or a common type of mutation. An additional mutation (marked as \* in Table 21) found in a sporadic case of HSAS first reported by Jouet et al., in 1994, is now presumed to be the result of gonadal mosaicism in the mother of the affected boy (Jouet and Kenwrick, 1995). The mutations which have been found in families presenting with combinations of MASA, SPG1 and HSAS are listed in Table 21.

Author	Type of mutation	Position	Amino acid change	Diagnosis
Fransen et al., 1994	C/T substitution	exon 28	Ser1194Leu	HSAS/MASA
Jouet et al., 1994	C/G substitution	exon 6	His210Gln	MASA
Jouet et al., 1994	2 bp deletion (TG)	exon 26	Frameshift/STOP	SPG1
Jouet et al., 1994	1 bp deletion (G)	exon 22	Frameshift/STOP	sporadic HSAS*
Vits et al., 1994	C/G substitution	exon 6	His210Gln	MASA
Vits et al., 1994	G/A substitution	exon 14	Asp598Asn	MASA
Vits et al., 1994	ORF deletion	exon 28		MASA
Jouet et al., 1995	G/A substitution	exon 8	Glu309Lys	MASA
Jouet et al., 1995	C/T substitution	exon 21	Pro941Leu	HSAS/MASA
Ruiz et al., 1995*	T/G substitution	exon 6	Ile179Ser	HSAS/MASA/SPG1
Ruiz et al., 1995**	G/A substitution	exon 9	Gly370Arg	HSAS/MASA

Table 21. Mutations which have been found in families presenting with MASA, SPG1 and HSAS or a combination of the three. \*This family was previously reported by Fryns et al., (1991). \*\*This family was previously reported by Kaepernick et al., (1994).

### 5.1.7 Carrier detection and prenatal diagnosis of HSAS

Since heterozygote carriers of HSAS are asymptomatic, carrier detection is usually not possible before the birth of an affected son. In families where there is a conclusive family history, HSAS can, in principle, be diagnosed prenatally by ultrasound during the second and third trimesters (Brocard et al., 1993). However,

this is unreliable because ventricular dilatation may occur late in pregnancy (Rogers and Danks, 1983) or even postnatally (Jansen, 1975), and adducted thumbs are observed in only 44 per cent of affected males (Halliday et al., 1986). In some families the age of onset of HSAS varies from one child to another (Holmes et al., 1973). Sonographic monitoring must be meticulous, repetitive and prolonged in order to diagnose HSAS prenatally.

Although prenatal diagnosis is possible by direct mutation analysis of *LICAM*, for most diagnostic laboratories this is not practical, unless a particular mutation has previously been identified in the family.

Family K was diagnosed with typical X-linked aqueductal stenosis before mutations in *LICAM* were identified as being the cause of the disease. DNA analysis was conducted using Xq28 markers which were known to be closely linked to the HSAS locus but the disease in this family appeared not to be linked to these markers.

5.2 FAMILY STUDIES

The X-linked hydrocephalus family (Family K) studied here is shown in Figure 46. The carrier female (III:3) had three affected boys who died perinatally with hydrocephalus and her sister (III:8) gave birth to a hydrocephalic boy (IV:7) who is still alive.

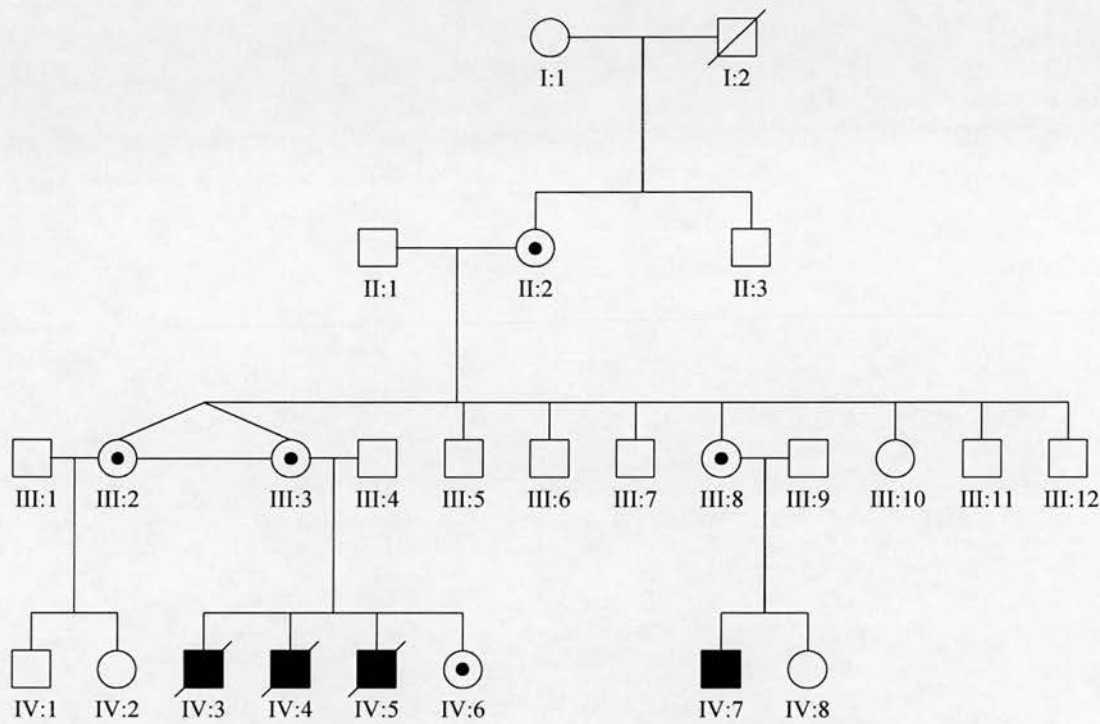


Figure 46. Pedigree of family K. Affected individuals are shown as filled black symbols and obligate carriers as symbols with a dot.

5.2.1 Case reports

Case 1 (IV.3) - 1973

The pregnancy progressed normally until 32 weeks' gestation, when the uterus was felt to be enlarged on examination. Abdominal X-ray at 34 weeks showed the fetal head to be grossly enlarged. Spontaneous labour occurred at 36 weeks and CSF was



drained by puncture at the time of delivery. Gross hydrocephalus was confirmed after delivery and the baby survived for about two hours. No further details were available.

#### Case 2 (IV.4) - 1974

The post-mortem examination of this baby was carried out by Dr J. Begg at Forth Park Maternity Hospital, Kirkcaldy.

At the end of this pregnancy Caesarean section was performed on account of massive hydrocephalus. The heart was beating at delivery but the child did not breathe and no resuscitation was attempted. At post mortem it was reported that "*there were abnormalities of the hands and fingers. There was massive dilatation of both lateral ventricles. The cortex overlying both was paper-thin. There was obviously some degree of obstructive hydrocephalus. The other major abnormalities in the head were the absence of olfactory nerves and the posterior fossae on each side of the base of the skull were poorly formed.*" The brain was not fixed.

#### Case 3 (IV.5) - 1980

The post-mortem examination of this baby was carried out by Dr A.D. Bain at the Royal Hospital for Sick Children, Edinburgh. The neuropathological study was carried out by Dr A. Gordon, Department of Neuropathology, Western General Hospital, Edinburgh.

Late in the pregnancy ultrasound scanning suggested hydrocephalus and labour was induced at 38 weeks. Caesarean section was performed after failure of the fetus to progress. The baby weighed 3.8 kg, OFC was 42.5 cm. He gasped at birth and Apgar scores were 3 at 1 minute and 3 at 5 minutes - no active resuscitation was given and regular respirations developed at 10 minutes. Examination showed a grossly hydrocephalic infant with generalised hypotonia and a single right palmar crease. The head transilluminated. The baby died 8 days later.



At post mortem, the brain was removed intact and fixed. *“There was marked symmetrical enlargement of both cerebral hemispheres which felt grossly cystic. The major gyral markings appeared to be present except in the parietal and occipital regions where the gyri were extremely broad. There was ballooning of the floor of the third ventricle. The section showed a very severe hydrocephalus which involved symmetrically all compartments of the lateral ventricles. There was gross bilateral ventricular dilatation, with only a few mm thickness of white matter overlying the convexities. There was no septum pellucidum and the corpus callosum was absent; being represented only by a thin membrane forming a ridge between the lateral ventricles. The temporal horns were less dilated than the remainder of the lateral ventricles. The anterior part of the third ventricle and the foramina of Monro were dilated. The basal ganglia were present but were slightly rotated outwards from the midline. The fourth ventricle, brain stem and cerebellum were normal, but the aqueduct was completely occluded. Histologically, the aqueduct was represented by an anterioposterior cleft with closely apposed walls, rather than the usual triangular shape, the resultant appearance resembling a zip-fastener, the cilia on opposite sides interlocking with one another. The section through the ventricular wall showed a reasonably normal cortex, a narrow sheet of white matter and a sub-ependymal layer of small round cells, with no visible lining epithelium.”*

#### Case 4 (IV.7) - 1990

The only surviving affected individual in this family was born in 1990 (Figure 47). Ultrasound scanning at 19 weeks gestation had shown no abnormality and elective Caesarean section was performed at term. The baby had obvious gross hydrocephalus. The birth weight was 4.7 kg, and apart from a brief apnoea lasting two minutes his breathing became well established and Apgar scores of 6 at one minute, 10 at five minutes and 10 at ten minutes were awarded. His OFC was 49 cm and he had a very large fontanelle and splayed sutures with prominent veins. At birth, cranial ultrasound showed enormous dilatation of the lateral ventricles, mild dilatation of the third ventricle and a normal fourth ventricle. The cerebellar vermis

was normal, and the cerebral cortex only 8 mm thick. His thumbs were noted to be adducted at birth but this was less apparent later in infancy and he had a normal pattern of palmar dermatoglyphics. He underwent an immediate ventricular tap from which pink CSF was obtained and at five days of age a ventriculo-peritoneal shunt with a right frontal Rickham reservoir was inserted. Cranial ultrasound at 9 weeks revealed absence of septum pellucidum and corpus callosum and CT scanning at age 8 months confirmed these anatomical abnormalities, with "*a complicated midline dysraphic problem with probable partial agenesis of the corpus callosum*". At the age of 10 months there was evidence of premature fusion of the sagittal and coronal sutures. Morcellation of the skull was performed in which bone was removed from the perisagittal suture and the coronal area. Formal psychological assessment at the age of 2 years placed the boy's performance in various areas in the 6-10 months range.

Therefore, the clinical and pathological investigations of affected males in Family K established an unequivocal diagnosis of X-linked aqueductal stenosis. Linkage analysis was carried out so that carrier females in this family could be offered future prenatal diagnosis. Unexpectedly, this resulted in negative LOD scores with markers which were previously shown to be linked to HSAS. For this reason, many other markers were analysed in distal Xq until positive linkage was eventually obtained with markers in Xq27.3.



Figure 47. Individual IV:7 at 8 months.

## 5.3 METHODS

### 5.3.1 DNA analysis

DNA was extracted from blood from all family members (except I:2, III:1 and the deceased individuals IV:3, 4, and 5) as described in 6.1.1.1. Paraffin embedded sections of brain were available from individual IV:5 but although I was able to extract DNA (6.1.1.5) from this tissue (it could be seen on an EtBr stained agarose gel), I could not PCR amplify the DNA with any CA repeat primers. The twins III:2 and III:3 were shown to be monozygotic by multilocus DNA “fingerprinting” using the hypervariable probe 33.15 (as described in 6.1.6).

Initially, markers in Xq28 (*DXS52*, *DXS15* and *F8C*) were analysed because these had previously been shown to be very closely linked to HSAS. Later, other markers within Xq28 and Xq27.3 were analysed. The markers which were analysed by blotting and hybridization (6.1.2 and 6.1.3) are shown in Table 22 and markers analysed by PCR are shown in Table 23. PCR reactions were carried out as described in (6.1.4) using either a radioactive method (6.1.4.a and 6.1.4.b) or a fluorescent method (6.1.4.c). Two exceptions were the *FMRI* CGG repeat which was carried out as in 6.1.4.1 and the *DXS52* VNTR which was carried out non-radioactively. In the latter system the repeat unit is approximately 60bp long and therefore alleles which differ by only one repeat can be distinguished on a 1% agarose gel stained with EtBr (Richards et al., 1991).

The initial results obtained in Family K with Xq28 markers (*DXS52*, *DXS1177*, *DXS15*, *F8C* and *DXS1108*) are shown in Figure 48 (page 177).

Locus	Probe	Enzyme	Alleles (kb)	Reference
<i>DXS152</i>	cX33.2	<i>Apa</i> LI	12,8.3	Arveiler et al., (1988b)
<i>DXS105</i>	55.E	<i>Pst</i> II	16,10	Mandel et al., (1986)
<i>DXS477</i>	2-34	<i>Taq</i> I	2.8,2.0	Rousseau et al., (1991)
<i>DXS465</i>	Do33	<i>Bgl</i> II	23,16	Rousseau et al., (1991)
<i>DXS296</i>	VK21A	<i>Taq</i> I	10.9,9.9	Suthers et al., (1989)
<i>DXS304</i>	U6.2	<i>Taq</i> I	7,3.3	Dahl et al., (1989c)
<i>DXS15</i>	DX13	<i>Bgl</i> III	5.8,2.8	Drayna et al., (1984)

Table 22. DNA probes analysed by radioactive hybridization. F814\* is a 'hybrid' probe which detects a *F8C Bcl*II diallelic polymorphism in addition to the VNTR at *DXS52*.

Location	Locus	Reference	Primers (5'-3')	Anneal temp.
Xq27.1	<i>DXS984</i>	Gyapay et al., (1994)	F TTTCTGTCTGCCAAGTGTTT R TACTGNGCCCTACTCCATTC	59°C
Xq27.3	<i>DXS691</i>	Lasser et al., (1993)	F TATGGGTAGGTTTGGGTTGA R GTTACACTCTTTCAGCCAGC	59°C
Xq27.3	<i>DXS548</i>	Riggins et al., (1992)	F AGAGCTTCACTATGCAATGGAATC R GTACATTAGAGTCACCTGTGGTGC	55°C
Xq27.3	<i>FMRI</i>	Fu et al., (1991)	F GCTCAGCTCCGTTTCGGTTTCACTTCCGGT R AGCCCCGCACTTCCACCACCAGCTCCTCCA	65°C
Xq27.3	<i>DXS1113</i>	Weber et al., (1993)	F ACCTGTGGAGGATAGTAGTCTGACT R GGGAGCTTTAGAGATTTTGGTAAAC	59°C
Xq28	<i>DXS52</i>	Richards et al., (1991)	F GGCATGTCATCACTTCTCTCATGTT R CACCACTGCCCTCACGTCACCTT	55°C
Xq28	<i>DXS1177</i>	Wehnert et al., (1993)	F GTATTATCAGCTAGCTGGGGCAGTC R GCGTCTTACGTTACCAGGATTAAGG	59°C
Xq28	<i>F8C</i> (IVS13)	Lalloz et al., (1991)	F TGCATCACTGTACATATGTATCTT R CCAAATTACATATGAATAAGCC	49°C
Xq28	<i>DXS1108</i>	Freije et al., (1992)	F GTGAATTCATCATATGTGATTTC R ACTAGGCGACTAATACAGTGGTGC	55°C

Table 23. Microsatellites analysed by PCR. The marker *DXS1177* was originally named p26 (Wehnert et al., 1993).

Other markers which were typed in Family K but which were uninformative (i.e. individual II:2 in Figure 48 was homozygous) were:

*CDR1*, *DXS98*, *DXS369*, *DXS297*, *DXS463*, *DXS305*, *F8C* (*HindIII* and *BclI* polymorphisms), and *DXS115*.

### 5.3.2 Linkage analysis

Two-point linkage analysis was carried out using MLINK. As in the previous two chapters, the disease segregating in this family is also reproductively lethal. An arbitrary mutation rate of  $1 \times 10^{-5}$  and a corresponding gene frequency of  $3 \times 10^{-5}$  were again chosen (assuming that male and female mutation rates are equal and the population is at equilibrium). Complete penetrance for the disease was assumed. The deceased individuals IV:3, IV:4 and IV:5 were coded as affected and the monozygotic twins (III:2 and III:3) were coded as one individual with two partners (because coding them as two individuals would artificially increase the LOD score by adding another informative meiosis). The alleles of each of the microsatellites were assumed to occur at equal frequencies and for the dimorphic markers, the published frequencies were used.

The results of the pairwise linkage analysis are shown in Table 24 (page 182). Multipoint analysis was also carried out as described previously (Chapters 3 and 4) before using the LINKMAP component of FASTLINK and genetic distances as shown in Figure 50. Fifteen overlapping five-point analyses (four marker loci and the disease locus) were carried out in this family. For the purposes of the LINKMAP analysis the mutation rates were set at zero. Genetic distances were converted into recombination fractions using the Kosambi mapping function prior to the calculation and reconverted to cM afterwards. Multipoint LOD scores were calculated from location scores and plotted against genetic distance (using the marker *DXS984* as the starting point). The final graph is shown in Figure 51 (page 183).



5.4 RESULTS

5.4.1 DNA analysis

The initial results obtained with Xq28 markers (*DXS52*, *DXS1177*, *DXS15*, *F8C* and *DXS1108*) in Family K are shown in Figure 48.

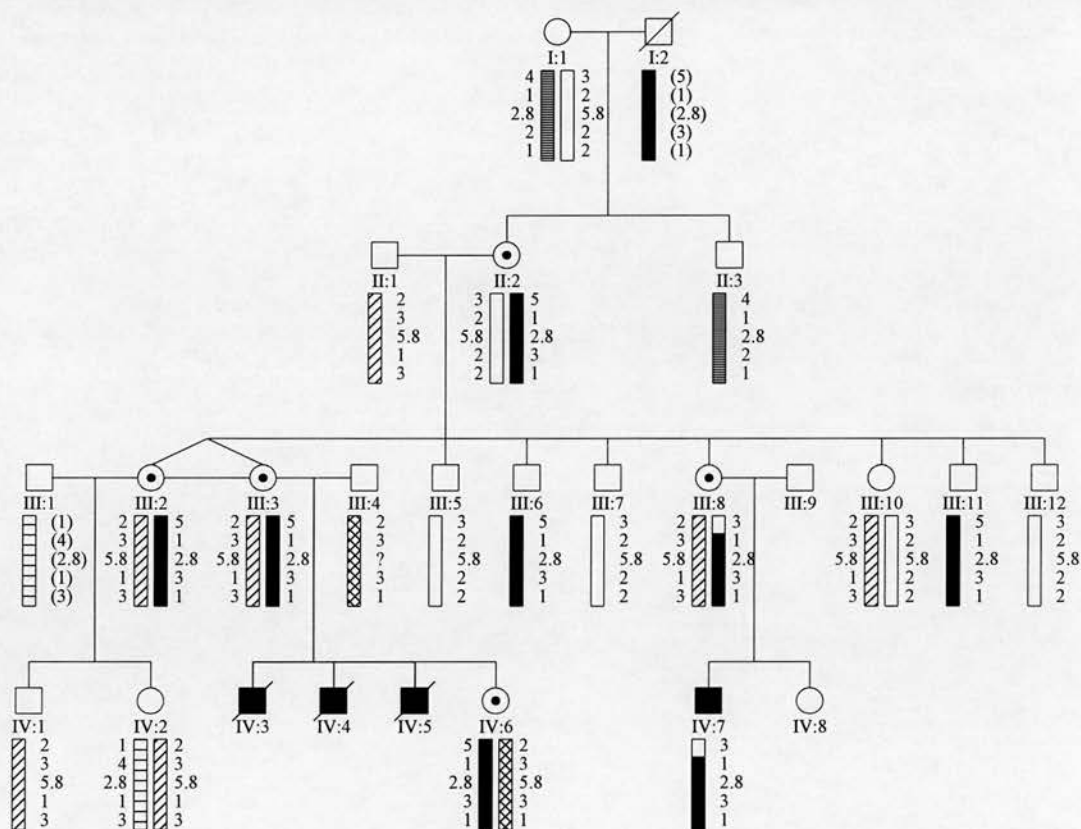


Figure 48. Pedigree of Family K showing haplotypes for Xq28 markers which had been shown to be linked to HSAS. Symbol designations are the same as in Figure 46. The markers are shown on the pedigree in chromosome order i.e. → Xter. Black bars have been arbitrarily assigned as the affected haplotype from individual IV:7.

It was apparent from the preliminary results (Figure 48) that the disease (which was undoubtedly X-linked aqueductal stenosis) in this family did not segregate with Xq28 markers. Two normal males in generation III have inherited the same Xq28 haplotype as the affected boy. To exclude the possibility of a sample mix-up, a fresh blood sample was obtained from each member of the family and the DNA analyses repeated, confirming the original results. The pedigree structure was also verified by multilocus fingerprinting using the probe 33.15, on both sets of samples. Furthermore, the affected boy has inherited the great-grandpaternal haplotype. Since linkage appeared to be excluded from this region, a search for linkage to other regions of the X chromosome was initiated. Twenty five markers were subsequently analysed in Xq28 and Xq27 and the 16 which were informative are shown on the pedigree in Figure 49.

In the construction of this pedigree, individual II:2 was taken as the starting point and the haplotypes of her offspring drawn as if II:2 had inherited a non-recombinant X chromosome from her mother (I:1). Haplotypes are drawn with markers in chromosome order (Xq27.1 to Xq28). For markers analysed by alkali blotting and radioactive hybridization, the actual sizes of alleles in kb are indicated on the pedigree; for CA repeats, alleles are denoted as numbers, beginning with 1 for the largest allele in the family; and for the *FMRI* CGG repeat, alleles are marked as repeat number. Inferred results are in brackets; if a result is unknown or cannot be inferred this is indicated by a "?". Six individuals in the pedigree are marked with a "+" - the significance of this will be discussed later.

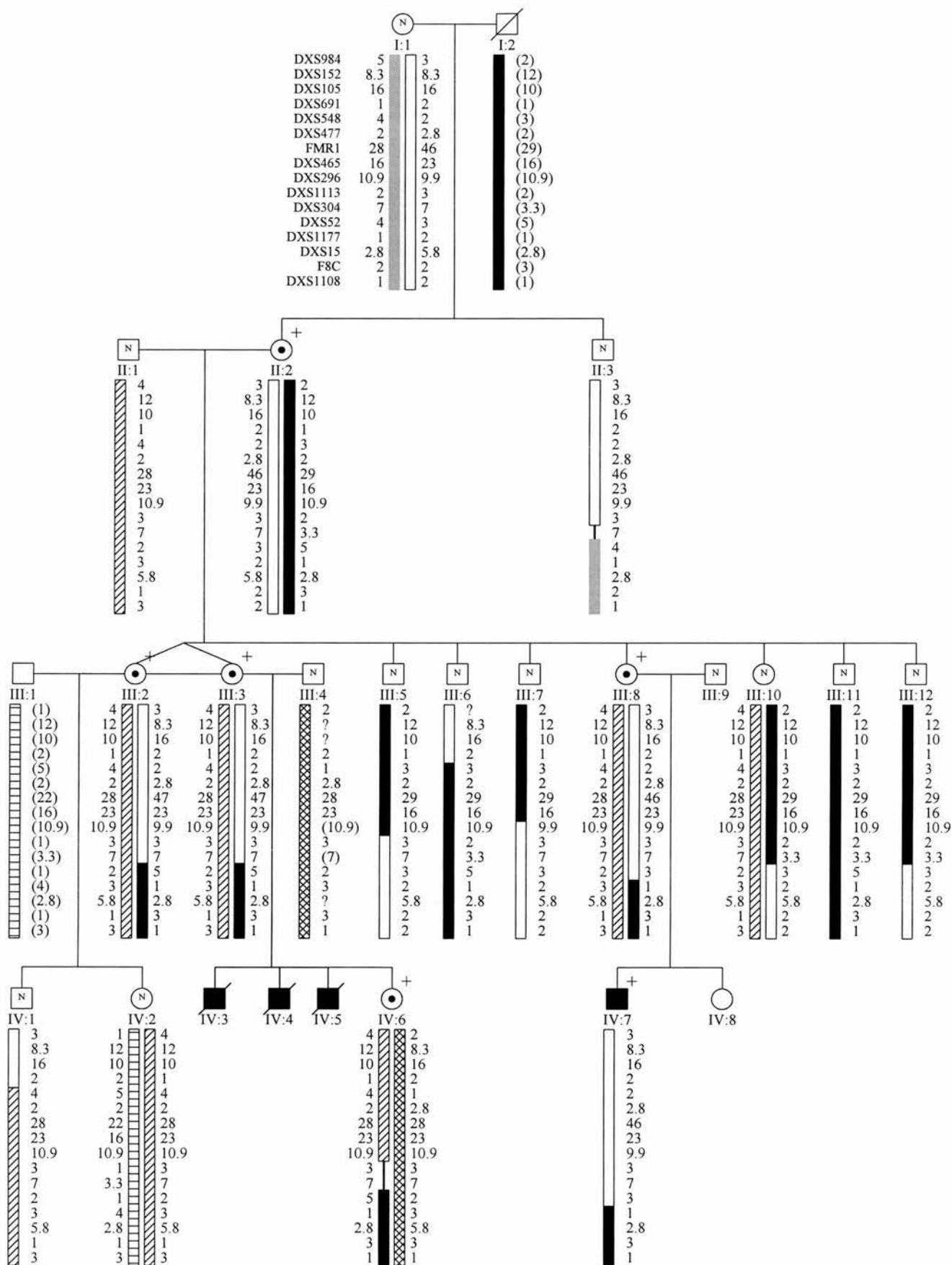


Figure 49. Results of DNA analysis in Family K. The structure of the pedigree, symbol designations and bar codes are the same as in Figure 48. See text for further explanation.

### 5.4.2 Linkage analysis

The results of the two point linkage analysis are shown as a LOD score table (Table 24) with maximum LOD scores ( $Z_{\max}$ ) at recombination fractions ( $\theta$ ). Only informative markers were used in the two-point linkage analysis. The result of the multipoint analysis is shown in Figure 51 (page 183).

Xcen ↑	cM	$\theta$
<b><i>DXS984</i></b>		
( <i>DXS152</i> )	3.5	0.035
<b><i>DXS105</i></b>		
( <i>DXS691</i> )	19.1	0.182
<b><i>DXS548</i></b>		
( <i>DXS477</i> )	0.3	0.003
<b><i>FMRI</i></b>		
( <i>DXS465</i> )	0.6	0.006
<b><i>DXS296</i></b>	0.7	0.007
<u><i>DXS1113</i></u>	1.5	0.015
<b><i>DXS304</i></b>	8.5	0.084
<b><i>DXS52</i></b>	0.9	0.09
<u><i>DXS1177</i></u>	0.22	0.002
<b><i>DXS15</i></b>	0	
( <i>F8C</i> )	2.1	0.021
<b><i>DXS1108</i></b>		
↓ Xtel		

Figure 50. Genetic distances between informative markers analysed in Family K. The order of markers was taken from Willard et al., (1994) and Fain et al., (1995). Figures on the left are genetic distances (cM) and were taken from a number of sources. Intermarker distances involving markers in bold were taken from Fain et al., (1995). Distances involving underlined markers were taken from the following: *DXS1113* (Weber et al., 1993); *DXS1177* (Wehnert et al., 1993). These were reported as physical distances and converted to cM on the assumption that 1Mb approximately corresponds to 1cM (Ott et al., 1991).

For the LINKMAP analysis these genetic distances were converted from cM to recombination fractions (shown on the right) using the Kosambi mapping function. Markers in brackets were excluded from the LINKMAP analysis because, either the genetic distances involving these markers were unobtainable, or the distances between two adjacent markers was zero.

There is some uncertainty about the exact location of the marker *DXS152*. According to the HGMP RC Genome Database it lies distal to *DXS105* but according to Willard et al. (1994) and Nelson et al. (1995b) it is as shown here. However, in this family this discrepancy is irrelevant because there are no recombinations between *DXS152* and the adjacent loci.

LOD scores at $\theta$ of:									
Locus	0.0	0.01	0.05	0.1	0.2	0.3	0.4	$Z_{\max}$	$\theta_{\max}$
<i>DXS984</i>	-2.54	-0.14	0.52	0.73	0.78	0.61	0.32	0.79	0.15
<i>DXS152</i>	-2.54	-1.43	-0.19	0.24	0.49	0.43	0.24	0.49	0.20
<i>DXS105</i>	-2.54	-1.43	-0.19	0.24	0.49	0.43	0.24	0.49	0.20
<i>DXS691</i>	-2.54	-1.77	-0.53	-0.05	0.26	0.25	0.11	0.28	0.25
<i>DXS548</i>	2.16	2.15	2.07	1.94	1.58	1.12	0.57	2.16	0.0
<i>DXS477</i>	2.16	2.13	1.97	1.77	1.32	0.81	0.27	2.16	0.0
<i>FMR1</i>	2.16	2.15	2.07	1.94	1.58	1.12	0.57	2.16	0.0
<i>DXS465</i>	1.56	1.55	1.48	1.38	1.11	0.75	0.33	1.56	0.0
<i>DXS296</i>	-2.54	0.15	0.78	0.97	0.95	0.73	0.37	0.97	0.10
<i>DXS1113</i>	-2.54	-2.19	-1.08	-0.56	-0.14	-0.01	0.01	0.01	0.35
<i>DXS304</i>	-2.54	-1.94	-0.75	-0.27	0.08	0.15	0.10	0.15	0.30
<i>DXS52</i>	-2.54	-2.51	-1.72	-0.96	-0.27	0.00	0.07	0.07	0.40
<i>DXS1177</i>	-2.54	-1.77	-0.53	-0.05	0.27	0.28	0.17	0.30	0.25
<i>DXS15</i>	-2.54	-1.77	-0.54	-0.07	0.23	0.25	0.14	0.26	0.25
<i>F8C</i>	-2.54	-1.78	-0.59	-0.15	0.11	0.11	0.06	0.12	0.25
<i>DXS1108</i>	-2.54	-1.77	-0.53	-0.05	0.27	0.28	0.17	0.30	0.25

Table 24. Results of two-point analysis in Family K for all informative markers (as shown on the pedigree in Figure 49).



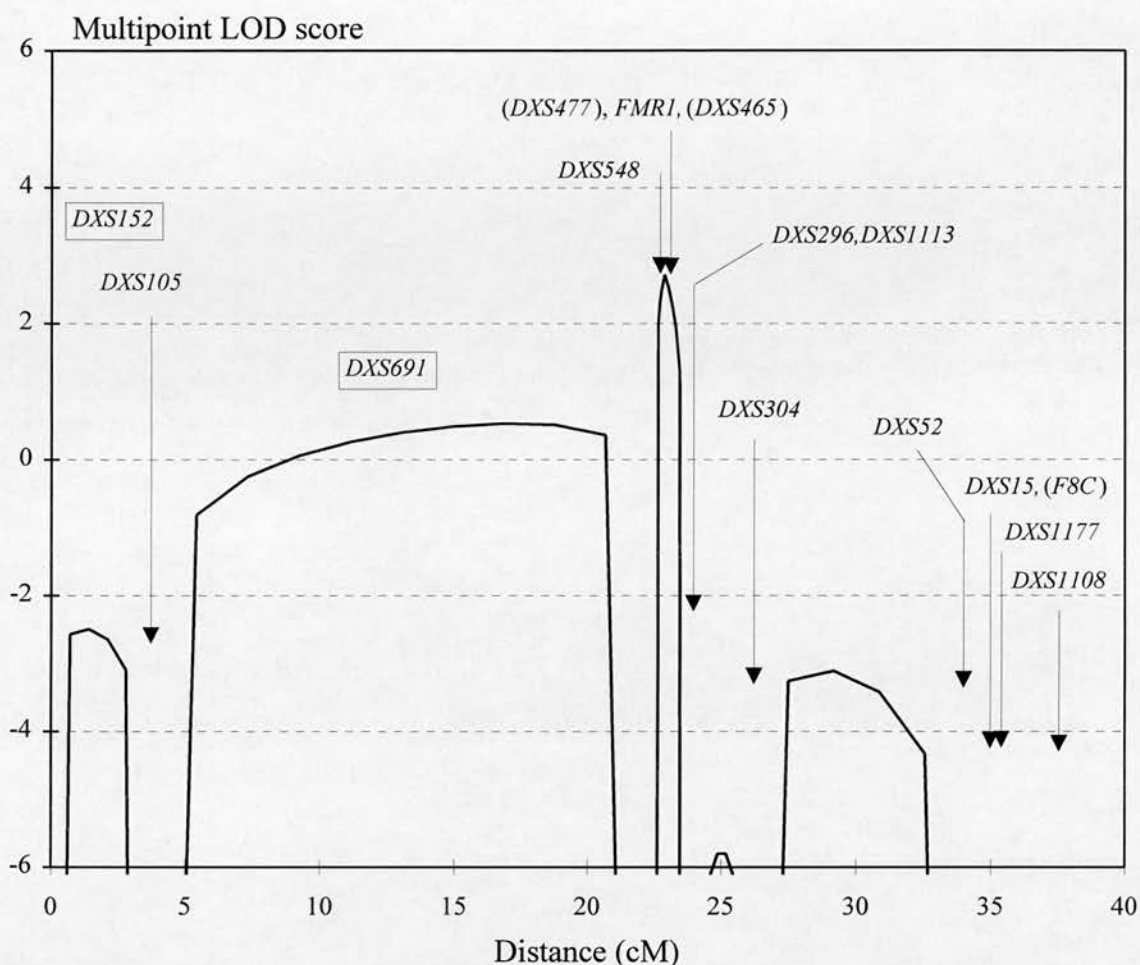


Figure 51. Results of the LINKMAP analysis in Family K using genetic distances as shown in Figure 50. The marker *DXS984* (not shown) was used as the starting point (0cM). As before, the location scores produced by LRP were converted to multipoint LOD scores and a negative LOD score cut-off point of -6 was chosen. The maximum multipoint LOD score obtained in this analysis was 2.71 with the microsatellite *DXS548* and the *FMRI* CGG repeat. Markers shown in brackets were excluded from the LINKMAP analysis for the reasons described in Figure 50. The two markers in boxes were also excluded from the analysis because their chromosomal locations are unknown. Ten evaluations were carried out in the interval between *DXS105* and *DXS548*.

### Conclusions so far

The clinical and neuropathological studies on the four males in this family have established an unequivocal diagnosis of “classical” X-linked hydrocephalus. However, linkage analysis using markers which are known to be closely linked to the *LICAM* locus gave negative LOD scores. Positive LOD scores were obtained with markers at, and close to, the *FMRI* locus. In addition, multipoint analysis also showed that the most likely location for the disease locus in Family K was at the *FMRI/DXS548* locus pair. These data led to the assumption that there must be a second gene for HSAS which is located in Xq27.3 and this was reported as such (Strain et al., 1994b). The putative second locus was named HSAS2. As expected, the possibility of genetic heterogeneity in HSAS caused some alarm among the genetics community because of the implications for prenatal diagnosis of the disease with linked markers (Serville et al., 1993). For this reason, we advocated that prenatal diagnosis of HSAS using linked markers should only be carried out in families which were large enough for linkage to HSAS1 to be demonstrated (Strain et al., 1994c).

### Identification of an *LICAM* mutation in Family K

Several months after the linkage analysis in Family K had been carried out, DNA from the living affected male (IV:7) was screened for a mutation in the *LICAM* gene by SSCP analysis (this was carried out by Monique Jouet and Susan Kenwick, Addenbrooke’s Hospital, Cambridge). A single mobility shift for exon 23 of the *LICAM* gene was detected (Jouet et al., 1996). Sequencing of PCR product of this exon revealed a single adenosine deletion at position 3088 within the L1 coding sequence. The effect of this would be the insertion of 72 novel amino acids and a premature stop codon (UGA) after amino acid 1029 of the mature protein. Heteroduplex analysis was carried out on all family members and six individuals were found to carry the mutation (marked with a cross on the pedigree in Figure 49). The heteroduplex analysis showed complete segregation of the mutation with the disease and proved that the exon 23 mutation is responsible for the hydrocephalus in Family K.

## 5.5 DISCUSSION

The possibility of genetic heterogeneity in HSAS could therefore eventually be refuted but explanations for the discordance between the mutation analysis and the linkage data in this family were sought. Three hypotheses are proposed:

### 1. Somatic and gonadal mosaicism (germline or germinal mosaicism) in II:2

The most probable (because it's the most parsimonious) explanation for the discrepancy in this family is that of somatic and gonadal mosaicism in II:2. Examination of the pedigree (Figure 49) shows that the affected Xq28 haplotype in II:2 (shown in black) has been derived from her father (I:2). As HSAS is reproductively lethal and the disease is fully penetrant, I:2 must be normal. This implies, therefore, that a *de novo* mutation has occurred in II:2 (on her paternally derived X chromosome). II:2 has passed on the same haplotype, with a mutation to the twins (III:2 and III:3) and to III:8, and without a mutation to her normal sons (III:6 and III:11). This suggests that she has two populations of gametes:- XX and XX<sup>+</sup>, where X<sup>+</sup> is the chromosome carrying the *LICAM* mutation. Furthermore, as the mutation was found in DNA from two separate samples of her peripheral blood (Jouet et al., 1996), II:2 must be a somatic as well as a gonadal mosaic, which indicates that the mutation occurred early in embryogenesis, before the partition of the gonadal and somatic cell lines (day 5-6 post conception).

Combined gonadal and somatic mosaicism has been reported elsewhere for a number of X-linked and autosomal diseases: haemophilia A (Bröcker-Vriends et al., 1990); haemophilia B (Sommer et al., 1995); Duchenne muscular dystrophy (Bunyan et al., 1994); Ehlers-Danlos syndrome (Kontusaari et al., 1992); retinoblastoma (Kato et al., 1994) and osteogenesis imperfecta (Bonaventure et al., 1992).

A similar situation to that which has occurred in Family K was reported by Arveiler et al. (1990) in a family with Wiskott-Aldrich syndrome. Linkage analysis using the

hypervariable marker *DXS255* (which is known to be closely linked to the WAS locus) gave negative LOD scores. This prompted a search for linkage to other regions of the X chromosome but when this proved fruitless, they considered the possibility of gonadal mosaicism in the grandfather of the affected boys. If this was the case, the WAS mutation would then be expected to be carried on the grandpaternal haplotype, although they could not prove this directly since the affected boys were dead. They were able to show, however, that the grandmother was not a WAS carrier by X-inactivation studies (WAS carriers have skewed X-inactivation).

In Family K, the possibility of gonadal mosaicism for the *LICAM* mutation in the maternal grandfather of the affected boy had been considered as an explanation for the negative LOD scores. However, this was ruled out because his Xq28 haplotype (*DXS52*, *DXS1177*, *DXS15*, *F8C*, *DXS1108*: 2, 3, 5.8, 1, 3) has been passed through the obligate carrier (III:2) to her normal son (IV:1). In addition, the Xq28 haplotype inherited by the affected boy (IV:7) is grandmaternal.

## 2. Two double crossovers

Apart from the discordant linkage data, one very unusual feature of the DNA analysis in Family K is obvious from examination of the haplotypes in Figure 49, and that is, there is an unexpectedly high frequency of recombination in this family irrespective of the segregation of the disease. In particular, there is 1 recombinant (III:5) between *DXS296* and *DXS1113* ( $\theta = 0.007$ ), 1 (III:6) between *DXS691* and *DXS548* ( $\theta < 0.182$ ), 1 (III:7) between *DXS465* and *DXS296* ( $\theta \approx 0.006$ ), 1 (III:8) between *DXS52* and *DXS1177* ( $\theta = 0.09$ ), and 2 recombinants (III.10, III.12) between *DXS304* and *DXS52* ( $\theta = 0.084$ ). A crossover has also occurred in IV:6 somewhere between *DXS296* and *DXS52* (her mother is homozygous at these loci so the exact region is unknown).

*LICAM* is situated between *DXS15* and *F8C*. A double recombination event in meiosis in II:2, within this interval, could move *LICAM* to the opposite chromosome

while maintaining phase between the flanking markers *DXS15* and *F8C* although such a recombination would have gone unnoticed. However, two such double recombination events would have to have occurred (giving rise to III:6 and III:11) in order to explain the pedigree data. However, no recombinations have been reported between these loci (and therefore the genetic distance is reported to be 0cM; Fain et al., 1995) which makes the probability of a double crossover within this interval infinitesimally small. Also, this possibility would still require the occurrence of a new mutation in spermatogenesis in I:2, since the affected haplotype for the *DXS1177-DXS1108* region is 1, 2.8, 3, 1, that carried on II:2's paternally derived X chromosome.

### 3. Chromosomal rearrangement

It is theoretically possible that a cytogenetic rearrangement in Xq28 has occurred in this family. Because of this, detailed cytogenetic analysis of the carrier female III.8 was carried out by Professor Christine Gosden (MRC Human Genetics Unit, Edinburgh). Fresh blood samples were subjected to high resolution banding which failed to reveal any abnormalities of the Xq27-q28 region (further details of the cytogenetic analysis are reported in Strain et al. (1994b)).

Although a rearrangement on one X chromosome is feasible, such an occurrence would be expected, if anything, to suppress local recombination, rather than to increase it. Furthermore, there is no evidence from the pedigree in Figure 49 of any double recombinations which one might expect if local chromosomal rearrangements had occurred. Multicolour FISH might be able to resolve whether there are any structural anomalies of distal Xq, although this would be extremely complex because, in order to space markers at 1Mb intervals, for example, 10 markers would be needed and would have to be used in multiple combinations.

Despite these residual uncertainties, the important issue in this family is that prenatal diagnosis can be carried out in future male pregnancies and this would simply be done by an *LICAM* exon 23 mutation detection.



One simple way of distinguishing hypothesis 1 from hypotheses 2 and 3 would be to analyse the family with an intragenic polymorphism. Under the first hypothesis, II:2 (if she was informative) would have passed the same allele to III:6 and III:11 as to the carrier women. On the other hand, if she passed the same allele to all the unaffected males and the opposite one to the carriers, this would support a local rearrangement. DNA from II:2 was analysed with the DNA probe p2HK13 (Willems et al., 1991) but she was uninformative. There is likely to be a CA repeat within *LICAM* but until recently only cDNA sequence was available. Now that the complete gene sequence is accessible at the HGMP resource centre it will be easy to search for a CA repeat within the gene.

The nature of the *LICAM* mutation found in this family is further supporting evidence that the single adenosine deletion is responsible for the disorder in Family K. Three other mutations have been identified in three independent HSAS families (also with severe phenotypes) which result in truncation of the L1 protein prior to the transmembrane domain (Jouet et al., 1996).

It is now clear that the negative LOD scores were obtained in this family because individuals III:6 and III:11 are normal males who carry the affected Xq28 haplotype. Equally, the positive LOD scores (2.16) obtained with *FMRI* and *DXS548* can be explained by the five normal sons of II:2 having inherited the opposite Xq27.3 haplotype to the one inherited by the affected boy (IV:7) and the obligate carrier females. The only discordant individual in this family is the normal individual (II:3) who has the same Xq27.3 haplotype as the affected boy (IV:7).

Finally, there were two interesting observations concerning the *FMRI* CGG repeat in this family. First, the disease appeared to segregate with a rare CGG repeat allele which is estimated to consist of 46 repeats. This is in the intermediate range (45-60) between normal and premutation alleles and according to our data (Chapter 2, Table 11), alleles of n=46 make up less than 0.2% of the total population. The second observation was that, in the meiosis which resulted in the twins (III:2 and III:3), the



CGG repeat has increased by one repeat to consist of 47 CGG triplets. This must have taken place very early in embryogenesis, or more likely as a meiotic event, as the change in size occurred in both twins (providing confirmation that they are monozygotic). For the linkage calculations this allele was assumed to consist of 46 CGG repeats.

The faint possibility that instability of the repeat could be involved in the disease was eliminated by Southern blotting using the probe StB12.3 on *Hind*III digested DNA (as described in **6.1.2** and **6.1.3**). This showed homogeneous, normal-sized bands in the affected boy (III:8) and his mother (IV.7).

## **6. Methods**

### **6.1 DNA ANALYSIS**

#### **6.1.1 DNA extraction**

##### **6.1.1.1 Peripheral blood and cord blood**

DNA was extracted from 5-20 mls of whole blood by an adaptation of two published methods. Initially, erythrocytes were lysed in a solution containing  $\text{NH}_4\text{Cl}$  (150mM),  $\text{NaHCO}_3$  (10mM) and  $\text{Na}_2\text{EDTA}$  (0.1mM), pH7.4 (Topic and Gluhak, 1991). The leucocyte pellet obtained after centrifugation and washing in this solution was resuspended in 3ml Tris-HCl buffer (50mM, pH7.4) containing NaCl (100mM) and  $\text{Na}_2\text{EDTA}$  (1mM). Proteinase K (100 $\mu\text{g}/\text{ml}$ ) and SDS (10mg/ml) were added and samples incubated at 37°C overnight. DNA was “salted-out” of this solution by the addition of 1ml of 6M NaCl as in Miller et al. (1988). DNAs were adjusted to a final concentration of 0.5mg/ml by the addition of TE buffer (10mM Tris-HCl, 1mM  $\text{Na}_2\text{EDTA}$ , pH8.0). DNAs prepared from peripheral blood did not usually require further purification by phenol-chloroform extraction. For DNA extraction from blood from young children, where the sample volume received was often less than 1 ml, the method was scaled down accordingly.

##### **6.1.1.2 Chorionic villi**

DNA was extracted from CVS (which had been sorted to remove maternal decidua) according to Old (1993). The villi were washed in 0.5ml of 150mM NaCl, 25mM  $\text{Na}_2\text{EDTA}$ , pH8.0, centrifuged briefly and the aqueous layer removed. The villi were resuspended in the same buffer with the addition of 0.1% SDS and 50 $\mu\text{g}/\text{ml}$  proteinase K and incubated overnight at 37°C. Two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and one chloroform/isoamyl alcohol (24:1) extraction were carried out. 250 $\mu\text{l}$  of 7.5M ammonium acetate was added to the final aqueous layer. This was mixed thoroughly and DNA precipitated by the addition of 1.5ml of

absolute ethanol. The DNA was washed with 70% ethanol, dried and dissolved in TE buffer.

In addition, for each CVS, three individual fronds of tissue were separated from the bulk of the cleaned tissue and placed in individual tubes. These pieces of tissue were washed in the above buffer and then treated exactly as for mouthwash samples (6.1.1.4). This provides material which can only be PCR amplified but is useful for obtaining a rapid preliminary result.

#### **6.1.1.3 Fetal blood**

DNA was extracted from fetal cord blood using a lysis buffer containing 10mM Tris-HCl (pH8.0), 320mM sucrose, 5mM MgCl<sub>2</sub>, and 1% Triton X-100, followed by incubation with Proteinase K (50 µg/ml) and SDS (0.1%) overnight at 37°C. The DNA was purified by phenol-chloroform extractions as for chorionic villi. The sucrose/Triton based buffer was used to lyse the fetal erythrocytes because they are resistant to lysis in the NH<sub>4</sub>Cl based buffer used on other blood samples.

#### **6.1.1.4 Mouthwash samples**

Mouthwash samples (each approximately 10ml) were centrifuged to pellet the buccal cells which were resuspended in 0.5ml 0.05M NaOH. This was transferred to a screw-cap 1.5ml tube and boiled for 20 minutes. 50µl of 1M Tris-HCl, pH7.7 was added and the tube centrifuged for 5 minutes to pellet debris. 3-5µl of the supernatant was used for PCR.

#### **6.1.1.5 Paraffin embedded tissue**

DNA was extracted from paraffin embedded tissue from an adaptation of a method described in Jackson et al. (1991). A 5µm tissue section was suspended in 100 µl of digestion buffer (100mM NaCl, 10mM Tris-HCl pH8.0, 25mM Na<sub>2</sub>EDTA, 0.5% SDS) and 0.1mg/ml of Proteinase K was added. This was incubated at 37°C for 48 hours. Two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and one

chloroform/isoamyl alcohol (24:1) extraction were carried out. DNA was precipitated by the addition of 10  $\mu$ l of 3M sodium acetate (pH5.2) and 2 volumes of cold ethanol and pelleted in a microcentrifuge at 13,000g. The DNA was dried and resuspended in distilled H<sub>2</sub>O. 1-5  $\mu$ l aliquots were used for PCR.

### **6.1.2 Alkali blotting**

5-10 $\mu$ g of DNA was digested with appropriate restriction endonucleases in a volume of 50 $\mu$ l and electrophoresed in 0.8% agarose gels in 1  $\times$  TAE buffer (0.04M Tris-acetate, 0.001M EDTA) containing 0.5 $\mu$ g/ml ethidium bromide. Gels were depurinated in 0.25M HCl and blotted on to Hybond N+ membrane (Amersham) in 0.4M NaOH. DNA probes were prepared by one of the following methods:

- i) Excision of the insert from the plasmid by digestion with appropriate restriction endonucleases. The insert and plasmid were separated by electrophoresis in 1.2% low melting point agarose and the agarose containing insert labelled directly without further purification.
- ii) Amplification across the insert with flanking PCR primers. PCR products were purified by adsorption onto glassmilk (Vogelstein and Gillespie, 1979).

### **6.1.3 Hybridization**

DNA probes (30-50 ng of insert) were labelled by the random priming method (Feinberg and Vogelstein, 1984) overnight at room temperature or for approximately 4 hours at 37°C. Hybridizations were carried out overnight at 65°C in a mix containing 5  $\times$  SSC (0.75M NaCl, 0.075M tri-sodium citrate), 5  $\times$  Denhardt's (0.1% PVP, 0.1% BSA, 0.1% Ficoll), 0.1% SDS, 0.1% sodium pyrophosphate, 10% dextran sulphate, and 100 $\mu$ g/ml sonicated denatured herring testes DNA. Filters were washed at 65°C to a final stringency of 0.5  $\times$  SSC and autoradiographed with intensifying screens for an average of 2 days.

#### 6.1.4 Polymerase chain reactions (PCR)

Unless otherwise stated all PCR reactions were carried out in 50  $\mu$ l volumes and contained the following ingredients:

	<u>Stock</u>	<u>Final concentration</u>
5 $\mu$ l dNTPs <sup>†</sup>	2mM	200 $\mu$ M of each dNTP
5 $\mu$ l 10 $\times$ buffer <sup>‡</sup>	10 $\times$	1 $\times$
1.25 $\mu$ l forward primer	10 $\mu$ M	0.25 $\mu$ M
1.25 $\mu$ l reverse primer	10 $\mu$ M	0.25 $\mu$ M
250 ng DNA		
1U <i>Taq</i> polymerase	5U/ $\mu$ l	
H <sub>2</sub> O to 50 $\mu$ l		

<sup>†</sup>deoxy nucleotide tri phosphates - consisting of equimolar amounts of dATP, dCTP, dGTP, dTTP. <sup>‡</sup>10  $\times$  PCR buffer is 100mM Tris-HCl (pH8.3), 500mM KCl, 15mM MgCl<sub>2</sub>.

For CA repeat polymorphisms, PCRs were carried out in one of three ways:

a) Incorporation of  $\alpha$ -<sup>32</sup>P-dCTP

2 $\mu$ Ci of  $\alpha$ -<sup>32</sup>P-dCTP (3000Ci/mmol) were added to the reaction mix as above prior to cycling. 2 $\mu$ l of product were added to 2 $\mu$ l of formamide loading dye (98% deionized formamide, 0.025% xylene cyanol FF, 0.025% bromophenol blue, 10mM EDTA pH8.0). Samples were denatured at 98°C for 5 minutes and electrophoresed in a 6% denaturing gel (6% acrylamide 29:1, 7M urea) at 70W in 1 $\times$  TBE buffer (0.089M Tris, 0.089M boric acid, 0.002M EDTA, pH8.0). The length of the run depended on the size of the product. The gel was dried without fixing and autoradiographed for 1-2 days.



b) 5' end labelling of one primer with  $\gamma$ -<sup>32</sup>P-ATP

Labelling reactions consisted of:

40 ng of primer per sample  
5  $\mu$ l (10  $\times$  PNK buffer<sup>†</sup>)  
4  $\mu$ l  $\gamma$ -<sup>32</sup>P-ATP (40  $\mu$ Ci)  
10U T4 polynucleotide kinase (PNK)  
H<sub>2</sub>O to 50  $\mu$ l

<sup>†</sup>10  $\times$  PNK buffer is 500mM Tris-HCl (pH7.6), 100mM MgCl<sub>2</sub>, 50mM dithiothreitol, 1mM spermidine HCl, 1mM EDTA (pH8.0).

These were incubated at 37°C for 1 hour followed by 10 minutes at 65°C to inactivate the enzyme. PCR reactions and electrophoresis were carried out as above.

c) Fluorescein tagged primer

One of the pair of PCR primers was conjugated at the 5' end with FluorePrime™ amidite (Pharmacia). This allows the addition of a linker bearing fluorescein to the 5' end of the oligonucleotide during the last step of automated synthesis on the Pharmacia Gene Assembler. PCR products were denatured in formamide loading dye (95% formamide, 20 mM EDTA and 10mg/ml Dextran blue 2M), and analysed on the automated laser fluorescent sequencer (A.L.F., Pharmacia) in a 6% denaturing acrylamide gel. Gels were run in 0.6  $\times$  TBE at 40W.

A size marker for A.L.F. was prepared by amplifying sections of the ApoB (Apolipoprotein B) gene in a multiplex PCR reaction. Primers were designed from published sequence so that the final product consisted of a ladder ranging from 100 to 500 bp in 50 bp increments. Two multiplex PCRs were required; one producing 100-200 bp fragments and the other producing 250-500 bp fragments. The forward primer in each case had been 5' end labelled with fluorescein during synthesis as described above.



All PCR reactions were carried out in a Perkin Elmer thermal cycler usually under the following cycling conditions:

94°C	5 mins		94°C	1 min			
x°C	1 min	⇒	x°C	1 min	⇒	72°C	10 mins
72°C	1 min		72°C	1 min			
(1 cycle)			(30 cycles)				

x = annealing temperature.

#### 6.1.4.1 PCR of the fragile X CGG repeat

PCR amplification across the CGG repeat was carried out essentially as in Fu et al., (1991) with the following modifications: 100-250ng of DNA was used in a final volume of 15µl, 10% glycerol was included, and the number of cycles increased to 35. 2µCi of  $\alpha$ -<sup>32</sup>P-dCTP was used per reaction and 2µl of product was electrophoresed in 5% denaturing sequencing gels in 1 × TBE at 70W for 3½ hours. Gels were dried without fixing and exposed as above.

The PCR primers used were (Figure 5)

**c** 5' GCTCAGCTCCGTTTCGGTTTCACTTCCGGT 3'

**f** 5' AGCCCCGCACTTCCACCACCAGCTCCTCCA 3'

An allele containing 30 repeats corresponds to a product of 308bp. The size marker used for the sequencing gels was pBR322 plasmid digested with *MspI* and end-labelled with  $\gamma$ -<sup>32</sup>P-ATP or  $\gamma$ -<sup>33</sup>P-ATP.

#### 6.1.5 PCR of the fragile X CCG repeat using a fluorescently labelled primer.

PCR was carried out as above using the same primers **c** and **f** except that primer **c** had been 5' end labelled with fluorescein during synthesis as described above. Samples were loaded onto A.L.F. as described previously.

### 6.1.6 Multilocus fingerprinting

This was carried out using the repeat unit multipriming (RUMP) method (Ferrie et al. 1991) to label the probe 33.15 (Jeffreys et al., 1985). DNA (2µg) was digested with *Hinf*I and electrophoresed in a 0.8% agarose gel in recirculated 1 × TAE for 48 hours. Alkali blotting was carried out as above. Filters were pre-hybridized in 0.5 M sodium phosphate buffer (pH7.2), 1% SDS at 62°C for 45 mins and then hybridized overnight at 62°C in 0.2M sodium phosphate buffer (pH7.2), 1% SDS, 1% BSA, 6% PEG 6000 and 0.5 ng/ml labelled probe. After hybridization, filters were washed twice (30 mins at 62°C) in 0.2 M sodium phosphate buffer (pH 7.2), 0.1% SDS and autoradiographed as before.

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## Appendix

# PRENATAL DIAGNOSIS OF FRAGILE X SYNDROME: MANAGEMENT OF THE MALE FETUS WITH A PREMUTATION

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## SUMMARY

Direct detection of the fragile X mutation by DNA analysis has greatly simplified prenatal diagnosis of this disease. However, women carrying a fragile X premutation may pass their expanded trinucleotide repeat to sons without expansion to a full mutation. Such sons are predicted to be intellectually normal. In this situation, the accuracy with which the fetal status can be inferred from analysis of chorionic villus sample (CVS) DNA is unclear. We describe such a case, in which it was felt necessary to proceed to fetal blood sampling despite technically unambiguous DNA results from the CVS. The lack of prospective data means that this dilemma may be expected to recur over the next few years when performing prenatal diagnosis on fragile X premutation carriers.

KEY WORDS—Fragile X, unstable DNA, premutation, fetal blood sampling, PCR, Southern blotting.

## INTRODUCTION

Fragile X syndrome (reviewed in Hirst *et al.*, 1992) is the commonest inherited cause of mental handicap. It is transmitted in an X-linked semi-dominant fashion, with females typically being more mildly affected than males. Affected males may show suggestive physical features (the Martin-Bell phenotype), consisting of long face and ears, rather coarse features, and post-pubertal macroorchidism. However, the phenotype may go unnoticed, and cannot be relied upon to make the diagnosis. Laboratory tests therefore play a crucial role in the clinical management of fragile X.

The fragile X phenotype is the consequence of inactivation of the *FMR1* gene, located at Xq27.3. This may result from deletions (Gedeon *et al.*,

1992; Wöhrle *et al.*, 1992) or point mutations (De Boulle *et al.*, 1993), but usually results from transcriptional silencing of the gene secondary to massive expansion of a trinucleotide repeat (CGG)<sub>n</sub> located within its first exon. Trinucleotide repeat expansion is accompanied by methylation of a CpG island around exon 1, somatic variability of the repeat length, and expression of the cytogenetic fragile site (*FRAXA*) from which the syndrome derives its name (Oberlé *et al.*, 1991; Verkerk *et al.* 1991; Fu *et al.*, 1991).

Normal individuals have a CGG repeat number  $n$  with  $6 < n < 46$  (Fu *et al.*, 1991). Affected individuals with fragile X have heterogeneous smears seen on Southern blots, generally reflecting  $n > 200$ . Between these limits is the so-called 'premutation' range (roughly  $50 < n < 200$ ). A premutation is not associated with abnormal CpG methylation, and males or females carrying premutations are phenotypically normal (Rousseau *et al.*, 1991). However, alleles in this range are unstable when transmitted from a carrier mother, usually undergoing a

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further increase in size. With females carrying large premutations, the probability of expansion to full mutation approaches 100 per cent, with an apparently sigmoidal curve determining the risk for smaller premutations (Fu *et al.*, 1991). Thus, when the X chromosome carrying a premutation is transmitted from a carrier mother, two general classes of outcome are possible: (i) expansion to full mutation or (ii) transmission of a premutation, often with some increase in size.

In this paper we describe a prenatal diagnosis in which a premutation was transmitted to a male fetus with an increase in size observed in CVS DNA, giving rise to considerable uncertainty as to the correct course of further management for the pregnancy. Because it was not felt possible on the basis of available data to be confident that the apparently homogeneous premutation reflected accurately the situation in the fetus, further fetal blood sampling was performed at 20 weeks' gestation. Direct analysis of fetal blood and subsequently of cord blood obtained at term failed to show any somatic instability.

## MATERIALS AND METHODS

### DNA analysis

DNA was extracted from blood by the method of Miller *et al.* (1988) and from cleaned CVS tissue according to Davies (1986). *Hind*III digests were performed on 5–10 µg of DNA, electrophoresed in 0.8 per cent agarose gels, and blotted onto Hybond N+ in 0.4 M NaOH. Hybridization to the intragenic probe StB12.3 (Rousseau *et al.*, 1991) was carried out overnight at 65°C in 5 × SSC, 5 × Denhardt's, 0.1 per cent SDS, 0.1 per cent sodium pyrophosphate, 50 per cent dextran sulphate, and 100 µg/ml sonicated denatured herring testes DNA. Filters were washed at 65°C to a final stringency of 1 × SSC and autoradiographed with intensifying screens for 12–48 h.

PCR amplification across the CGG repeat was carried out as in Fu *et al.* (1991) using 2 µCi of [ $\alpha^{32}$ P]dCTP per reaction and electrophoresis of 2 µl of product in 5 per cent denaturing sequencing gels at 70 W (40 V/cm) for 3½ h. Gels were dried without fixing and exposed as above.

### Cytogenetic analysis

Conditions for fragile X analysis of fetal blood were as described by Gosden *et al.* (1992). Fragile

X analysis of the CVS was carried out as in Rooney and Czepulkowski (1992).

## RESULTS

### Case report

The patient (II.12), a 23-year-old primigravida, was referred to the genetics clinic at 8 weeks' gestation because of a family history of fragile X syndrome. Her family (Fig. 1) had been investigated 4 years previously, but lost to follow-up. At that time, the patient and her three sisters had been given low risks of carrying the fragile X gene, on the basis of failure to demonstrate fragile sites in all tested family members except the proband III.1. (III.3 had not been tested at that time.) The family was reanalysed by Southern blotting and PCR, with the results shown in Fig. 1.

The presence of two normal CGG alleles in the mother (I.2), both of which had been passed to normal sons, confirmed that the dead father (I.1) was a normal transmitting male who had passed a premutation to each of his four daughters (Fig. 2, lanes 3, 4, 7 and 9).

After discussion, II.12 chose to undergo transabdominal CVS at 10 weeks' gestation. A male fetus was demonstrated by PCR of the pseudo-autosomal boundary region of the Y chromosome (Ellis *et al.*, 1990) and Southern blotting indicated the presence of an apparently homogeneous premutation (Fig. 2, lane 10). On PCR, this was found to correspond to an allele which had increased in size from 74 to approximately 106 repeats (Fig. 3a). Analysis of cultured chorionic villus tissue showed a 46,XY karyotype with no evidence of fragile sites in 100 cells cultured in folate-deficient medium.

On the basis of these data, a low risk of mental handicap was given, and the pregnancy continued. It was not felt possible, however, to exclude mosaicism completely in this pregnancy, and therefore our patient elected for fetal blood sampling at 20 weeks.

Kleihauer testing indicated a sample of 100 per cent fetal origin. Southern blotting and PCR of fetal blood DNA (Fig. 3b) again indicated a homogeneous premutation. Cytogenetic analysis of 170 lymphocytes cultured under thymidylate stress failed to show any evidence for fragile sites.

At term, DNA was prepared from cord blood. A premutation was again seen on PCR analysis



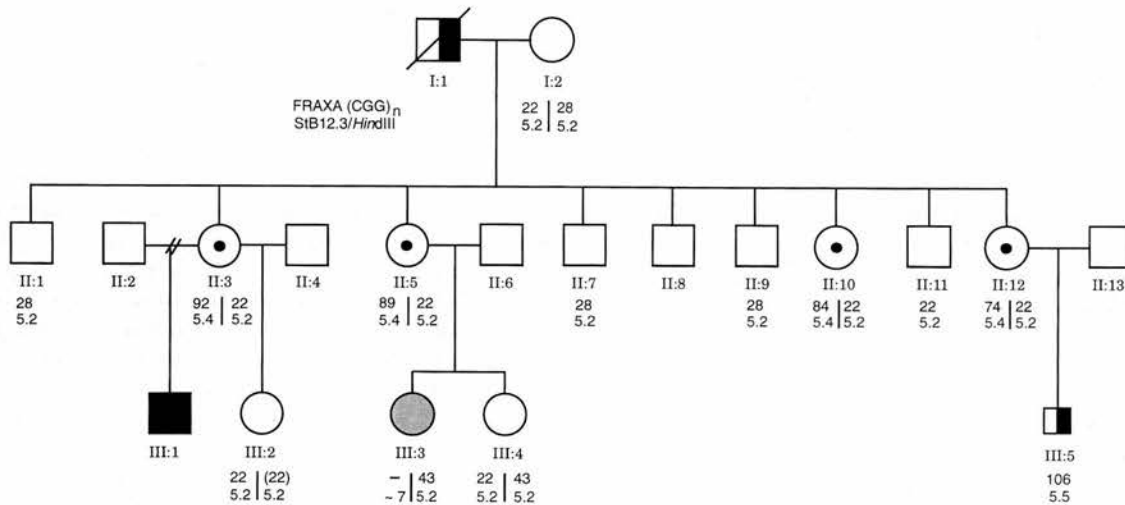


Figure 1—Pedigree of family. Affected males are indicated as ■, female premutation carriers as ○, female carriers of a full mutation as ●, and male premutation carriers as ▨. FRAXA (CGG)<sub>n</sub> alleles are denoted as number of repeats, and StB12.3 alleles as sizes in kb

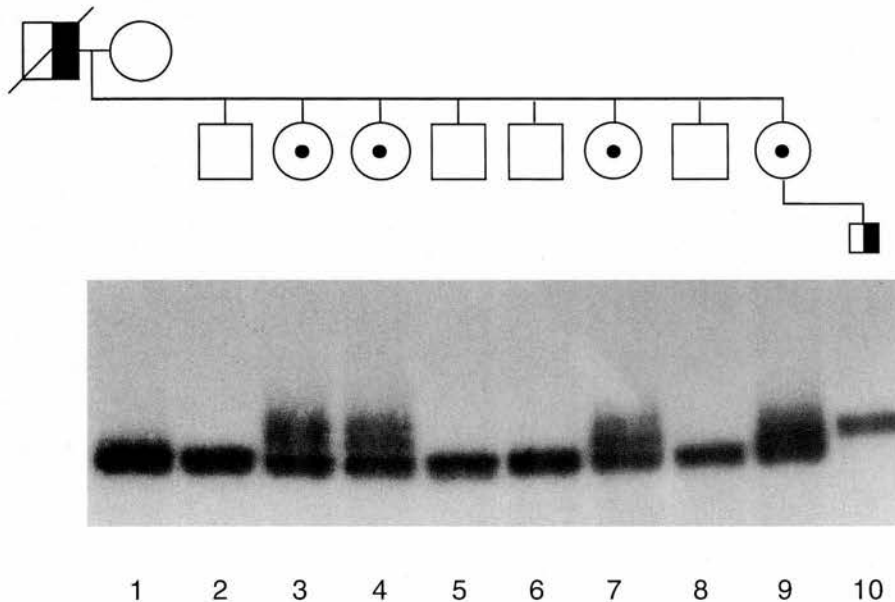


Figure 2—Southern blot of *HindIII*-digested DNA probed with StB12.3

(Fig. 3c) and Southern blotting (Fig. 4a). The premutation size in CVS and cord blood DNA appeared identical on PCR. Additional digests of the cord blood DNA were performed with *HindIII* and the methylation-sensitive enzyme *BstZI*. These showed only an unmethylated 3.1 kb band

(Fig. 4b), slightly larger than the corresponding maternal fragment, confirming that the CpG island at the 5' end of the *FMR1* gene was not hypermethylated. Culture in folate-deficient medium failed to show fragile sites in 100 cells analysed.

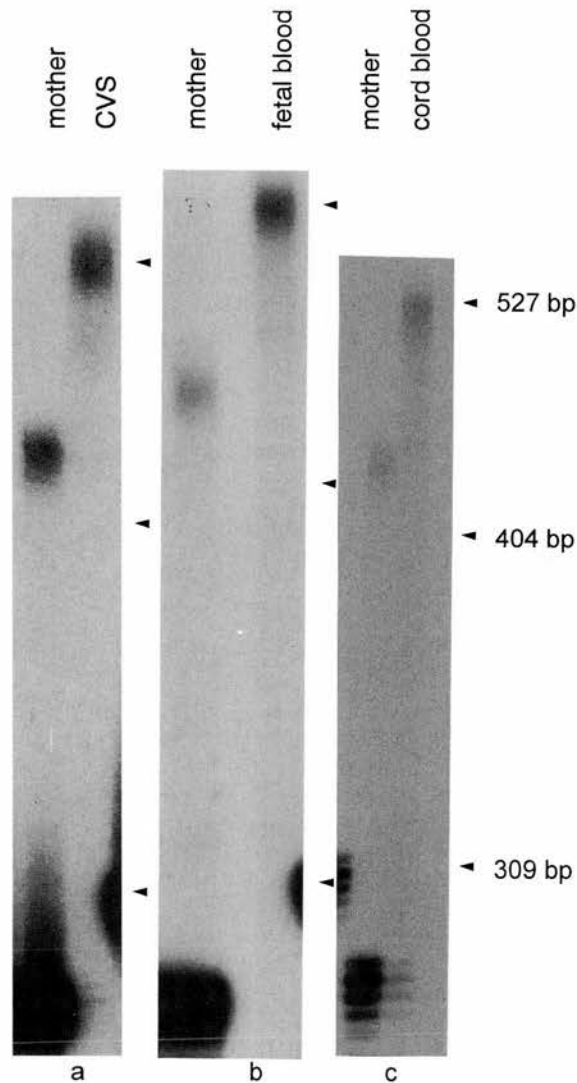


Figure 3—PCR amplification of CVS DNA, fetal blood DNA, and cord blood DNA. Size marker is pBR322 digested with *MspI*

## DISCUSSION

Molecular genetic linkage studies have been important for the prenatal diagnosis of fragile X for some years; analysis of the segregation of closely linked polymorphisms (Riggins *et al.*, 1992; Richards *et al.*, 1991) can still predict with a high degree of accuracy whether the high-risk *FMRI* allele in a family has been inherited by a fetus, but when the mother is a premutation carrier, it does not distinguish between a fetus carrying a

premutation or full mutation. Direct analysis of the fragile X mutation (Rousseau *et al.*, 1991; Fu *et al.*, 1991), in contrast, in addition to eliminating the small possibility of error due to recombination, permits pregnancies such as the one described here to continue, though they might previously have been terminated on the basis of a DNA linkage result. This advance, though, critically assumes that a normal outcome can be predicted from the finding of a premutation in CVS tissue.

While there is no reason to question the underlying assumption of a normal outcome for a fetus inheriting a premutation, the scantiness of current data does suggest caution in the interpretation of prenatal tests on CVS material. Some males with fragile X syndrome are so-called 'mosaics', with a population of premutation-carrying cells in addition to those with the full mutation (Rousseau *et al.*, 1991; Nakahori *et al.*, 1991). While the premutation-carrying cells in such males might perhaps arise from contraction of repeats previously in the full mutation range, the alternative possibility must be considered, of transmission in the zygote of a premutation, followed by a major role of mitotic instability in generation of the population carrying full mutations. Such post-conceptual instability could in fact be the sole mechanism of production of full fragile X mutations; this instability probably only operates during a small early window of embryonic development (Wöhrle *et al.*, 1993; Reyniers *et al.*, 1993). However, its occurrence raises the worrying possibility that the distribution of full mutation and premutation could differ from one tissue to another. This point is crucial for prenatal diagnosis, when usually DNA from extraembryonic tissue (chorionic villus) is analysed for the presence of the mutation. The mechanism or timing of mitotic instability may differ in chorionic and embryonic tissue, and therefore it is possible that a large premutation appearing stable in CVS DNA may have undergone greater expansion and somatic instability in the embryo. There is little published data bearing on this issue, but quantitative differences in the distribution of pre- and full mutation between CVS and fetal DNA have been noted by Devys *et al.* (1992), suggesting that there are indeed grounds for caution.

### General implications for fragile X prenatal diagnosis and screening

A mother's CGG repeat number  $n$  determines the risk of expansion to full mutation. Although

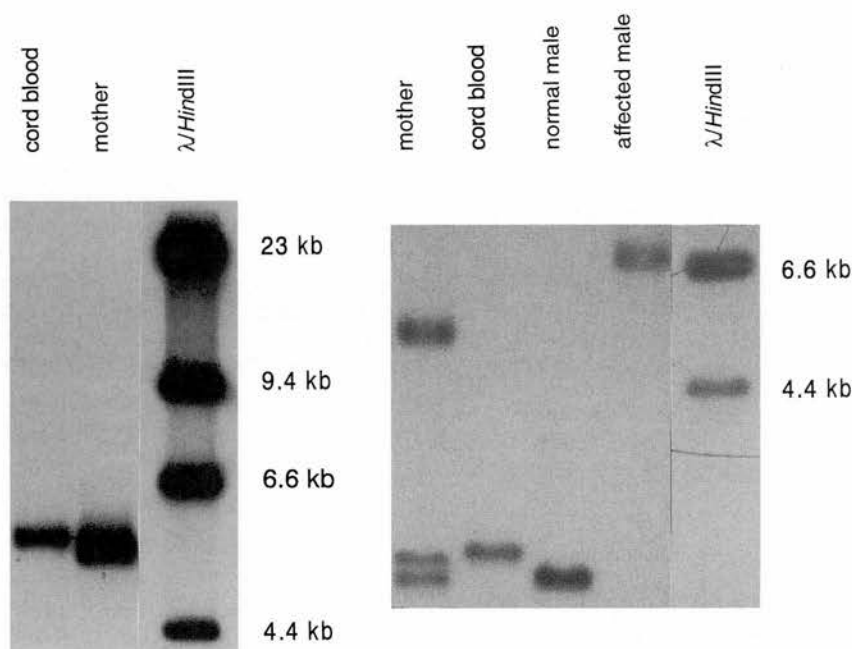


Figure 4—Southern blot of *Hind*III-digested DNA (a) and *Hind*III/*Bst*ZI-digested DNA (b) probed with StB12.3

the absolute value of the risk attached to each allele size has been questioned on the grounds of biased ascertainment (Bundey and Norman, 1993), it is clear that alleles of <50 repeats are very stable and those >200 very unstable in maternal transmission. The overall sigmoidal shape of the curve relating allele size to risk (Fu *et al.*, 1991) is therefore likely to hold true, and more precise absolute risks should be obtainable by formal segregation analysis of families to allow for proband effects. In general, the smaller the mother's premutation, the greater will be the chance of the fetus inheriting a premutation rather than a full mutation. This makes the relationship between premutation CGG repeat number in CVS and embryonic tissue a crucial one, which needs to be established for a large number of sample pairs. Additional reports of the outcome of pregnancies, in which a premutation has been transmitted, are therefore required. Because of present uncertainty over the confidence with which fetal premutation size can be inferred after the finding of a premutation in CVS, we currently recommend fetal blood sampling for further DNA analysis in this situation. We have already pointed out that this approach, if it proves not to be overcautious, has major implications for population screening for

fragile X (Bonthon and Strain, 1993); it implies that a substantial proportion of the mothers detected as premutation carriers by any screening programme may have to proceed to fetal blood sampling.

#### ACKNOWLEDGEMENTS

We are grateful to Kypros Nicolaides (King's College Hospital) for carrying out the fetal blood sampling. This work was supported by the Ludovici Bequest Fund to the University of Edinburgh and the Scottish Home and Health Department.

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The cadaveric growth hormone is available on the black market and is about half the price of recombinant growth hormone. The source of the cadaveric growth hormone is the former Soviet Union where pituitary-derived growth hormone is still produced. We presume that this group of growth hormone recipients is not adequately informed about the potential risk for Creutzfeldt-Jakob disease and the possible risk of transmission by tissue or organ donation.

We had the opportunity to analyse a growth hormone ampoule from the former Soviet Union. The specific activity was low (about 2 U/mg protein) and on sodium dodecylsulphate gel-electrophoresis was not pure monomeric, which jeopardises the quality of this hormone. It appears unlikely that suitable precautions were taken to eliminate slow virus contamination.<sup>3</sup>

Because this type of growth hormone is used in other countries, (anecdotally we know of use by weight lifters and body builders in Germany, Poland, Italy, and Austria), more information should be given to potential users.

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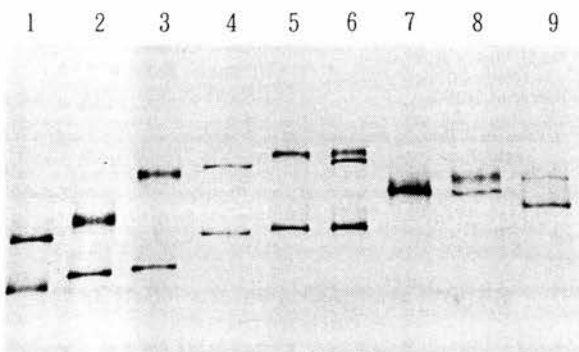
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### DNA single-strand conformation polymorphism method to distinguish DR4 alleles

SIR,—Mr Young and Dr Darke (Jan 16, p 183) report that DNA single-strand conformation polymorphisms (SSCPs) are not ideal for predicting HLA compatibility for the DR4 group, and these findings contradict those of Summers and colleagues.<sup>1</sup> We have used our established polymerase chain reaction (PCR)-SSCP method for DRB1 alleles (unpublished) to examine DR4 alleles.

Eight different DRB1 alleles of the DR4 group from Japanese, Korean, and Russian (DRB1\*0401, 0402, 0403, 0404, 0405, 0406, 0407, and 0410) individuals were identified by the PCR-SSO (sequence-specific oligonucleotide) method<sup>2</sup> or by direct sequencing. SSCP banding patterns are known to vary with the conditions of electrophoresis, especially gel concentration and



SSCP patterns of HLA-DRB1 alleles in DR4 group.

DRB1 gene exon 2 fragments of HLA-DR4 alleles were selectively amplified by PCR with primer pair DRBAMP-4 and DRBAMP-B.<sup>3</sup> 1 µL of amplified solution was mixed with 7 µL of denaturing solution (95% formamide, 20 mmol/L edetic acid [EDTA], 0.05% bromophenol blue, and 0.05% xylene cyanol FF), heated at 95°C for 5 min, and applied to a 10% polyacrylamide gel (acrylamide/bisacrylamide = 49/1, 5% glycerol). Electrophoresis was done in 45 mmol/L tris-borate (pH 8.0) and 1 mmol/L edetic acid at 4°C, followed by silver staining. Genomic DNAs were obtained from the following blood samples: (1):DRB1\*0407/0803; (2):0401/1502; (3):0405/-; (4):0403/1201; (5):0406/0901; (7):0404/0101; (8):0410/1502; (9):0402/-; (6):mixed sample of PCR products obtained from (4) and (5).

temperature.<sup>3</sup> Furthermore, we found that application of a small amount of amplified DNA to non-denaturing electrophoresis followed by silver staining resulted in distinct banding patterns. All examined DR4 alleles were discriminated both at 4°C (figure) and at 22°C (not shown), including DRB1\*0403 (lane 4) and 0406 (lane 5), which were not discriminated with the conditions used by Young and Darke. Lane 6, a mixture of DRB1\*0403 and 0406, discriminated these alleles clearly. All tested alleles of DRB1 in addition to DR4 were discriminated in our system.

This method can be used not only to discriminate alleles but also to confirm the donor-recipient molecular matching in transplantations.

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### Population screening for fragile-X syndrome

SIR,—Fragile-X mental retardation syndrome is in many ways, as stated by G. E. Palomaki and Dr Haddow (Feb 6, p 373), an ideal target for prenatal screening. Although we, like other UK genetic units, are considering a fragile-X screening programme, we cannot agree that "the only significant barrier, at present . . . is the variability in the severity of mental retardation in female offspring with the full mutation". Palomaki and Haddow ignore important aspects of fragile-X syndrome that will complicate prenatal screening.

When a premutation is found in a mother, there are a number of possible outcomes when the affected chromosome is transmitted. First, expansion to full mutation may occur. This creates problems of interpretation in a female fetus because the intellectual outcome is so variable. While our ability to correlate intellectual outcome to the results of methylation and other DNA studies might improve, this may not help prenatal diagnosis. For example, if tissue-specific variation in the pattern of X-inactivation proves an important determinant of intellectual outcome, analysis of amniocytes, or extra-embryonic material obtained at chorionic villus sampling (CVS) would be unlikely to yield useful information. Those considering prenatal screening for fragile-X may still have to communicate a 50% risk of mild mental handicap to mothers carrying female fetuses with a full mutation.

Second, transmission of a premutation may occur, often with some increase in size. This should result in a normal outcome, but caution is needed in interpreting test results, particularly on CVS material. The existence of affected "mosaic" males, who have cells carrying premutations and cells carrying full mutations, underlines the need to consider somatic instability of the fragile-X repeat. The process of instability development may not be the same in chorionic and embryonic tissue: a large premutation which appears to be stable on CVS analysis may have undergone greater expansion and somatic instability in the embryo. Little has been published on this issue. However, we have experience of one prenatal diagnosis in which the degree of somatic instability of a full mutation seen on chorionic villus tissue was much less than that seen in all fetal tissues recovered after termination. We currently recommend fetal blood sampling for further DNA analysis on male fetuses shown to be carrying large premutations on CVS. This approach may prove to be overcautious, but it implies that a substantial proportion of the mothers detected as premutation carriers by a screening programme may have to proceed to fetal blood sampling.

Third, the risk at which a prenatal test is offered needs to be defined. A sigmoidal curve relates a mother's CGG repeat number



$n$  to the risk of expansion to full mutation.<sup>1</sup> These data may become more precise as greater numbers of families are analysed. However, the lower the cut-off value of  $n$ , the greater will be the number of pregnancies in which the fetus appears to have inherited a premutation rather than a full mutation. Thus, the true risks attached to finding a premutation at prenatal diagnosis must be defined before setting a value of  $n$  at which testing is offered.

In addition to these biological uncertainties, there are technical problems hindering implementation of prenatal screening for fragile-X. These have prevented us from adding fragile-X into the framework of the Edinburgh trial of cystic fibrosis screening in antenatal clinics.<sup>2</sup> Rapid diagnosis by polymerase chain reaction of premutations<sup>1</sup> on mouthwash samples is in our experience simple and cheap, but full mutations do not amplify, and full mutation carriers are not distinguished from the 10–15% of women who are homozygous for allelic (CGG)<sub>n</sub> variants within the normal size range. These full mutation carriers are at the highest risk of having mentally handicapped offspring, and therefore must be detected by any programme aiming at high sensitivity. The only reliable detection method at present involves use of blot hybridisation. This is labour-intensive, skilled, and expensive to perform on several thousand samples annually.

None of these difficulties is insuperable. However, population screening for fragile-X will undoubtedly be more complicated than suggested by Palomaki and Haddow. There is a need for trials in several centres, involving different protocols and analytical methods, before a consensus can be reached over feasibility and desirability of fragile-X screening.

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SIR,—There is a major flaw in G. E. Palomaki and Dr Haddow's argument about screening for the fragile-X premutation. They suggest that nearly half the pregnancies of women carrying the premutation would result in a child with the full mutation. However, this figure is based on data obtained from families ascertained through an affected child, and as such is inevitably biased. The genetic risk for a woman carrying the premutation and with no family history of mental retardation is not known. However, genealogical studies from Sweden, Finland, and the Netherlands<sup>1–3</sup> show that premutation can exist in a family for many generations. Moreover, studies of the AC repeat sequences that flank the fragile-X gene show that certain haplotypes are associated with increased size of the CGG repeat of the gene, even in normal individuals, providing evidence for only a few ancestral chromosomes.<sup>4</sup> The risk for a premutation expanding to a full mutation could be very much less than 1 in 2. More information is needed on the definitions of the normal gene, the stable premutation, the unstable premutation, and the genetic significance of the last two. Until these are known, consideration of screening healthy women in the population is unwisely.

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SIR,—We agree with G. E. Palomaki and Dr Haddow that fragile X syndrome has the high frequency and morbidity necessary to make carrier screening cost-effective from a societal viewpoint, and that the test for expansion of the (CGG)<sub>n</sub> repeat region near the *FMR-1* gene is highly sensitive and specific. However, we feel that certain factual statements related to clinical and laboratory genetic aspects of this condition are potentially misleading. We also disagree with their final conclusion.

It is correct that among the unaffected population, only female individuals need carrier testing if the intent is to discover people at risk of having affected children. Unaffected male carriers have premutations that will be inherited by none of their sons and all of their daughters, who will be unaffected. (Affected males have full mutations and, although they are usually fertile, generally do not reproduce.) However, in females it is inaccurate to equate the terms carrier with premutation, and affected with full mutation. Unaffected female carriers ascertained by DNA analysis would include women with either a full or a premutation. Full mutations remain full if passed to the next generation, whereas premutations have a probability of becoming full that depends on the actual length of the DNA expansion, and can reach 100%. Males that inherit full mutations are nearly always retarded, and about half the females that do so are retarded.<sup>1</sup>

If the mother is identified as having a premutation or full mutation, then fetal testing is available. Fetal cells from chorionic villus sampling (CVS) or amniocentesis can be used but CVS offers the advantage of earlier detection. Despite the reliability of direct DNA analysis, simultaneous cytogenetic analysis for the presence of the fragile X site is recommended. The combined data are important in the final interpretation of the risk for affected versus carrier offspring, especially in the female fetus. A range of observed outcomes and probabilities would be given, allowing the parents to make an informed decision.

DNA-based fragile X screening of pregnant women with a suggestive family history in their extended families has been initiated by at least two groups,<sup>2,3</sup> who reported screening results for 103 and 40 women, respectively. 2 pregnant carrier females were identified in the 103 family study, with no previous known history of fragile X. This group of patients, at least, should continue to be offered screening for fragile X. However, a positive family history is not essential for a female to be a carrier. In the past 6 months in our prenatal clinics, several pregnant women with a negative family history have requested fragile X screening. We support the test being offered to informed patients, most of whom would have clearcut test results, and advocate fragile X testing more broadly to members of the prenatal female population.

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### Simple analgesics versus NSAIDs for osteoarthritis

SIR,—Professor Dieppe and colleagues (Feb 6, p 353) correctly note the lack of peer-reviewed clinical trials of simple analgesic versus non-steroidal anti-inflammatory drugs (NSAIDs) in patients with osteoarthritis, and suggest that the absence of such studies can be attributed to the fiduciary interests of the pharmaceutical manufacturer.

In describing the very few studies that provide this comparison these workers refer to our comparison of patients with osteoarthritis of the knee treated with ibuprofen 1.2 g or 2.4 g daily or paracetamol 4 g daily.<sup>1</sup> Their table summarising our results indicates that ibuprofen, in comparison with paracetamol, significantly improved pain at rest. It is misleading, however, to summarise the results of our study by that point alone. All 3 of our treatment groups showed



## Genetic Heterogeneity in X-linked Hydrocephalus: Linkage to Markers within Xq27.3

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### Summary

X-linked hydrocephalus is a well-defined disorder which accounts for  $\geq 7\%$  of hydrocephalus in males. Pathologically, the condition is characterized by stenosis or obliteration of the aqueduct of Sylvius. Previous genetic linkage studies have suggested the likelihood of genetic homogeneity for this condition, with close linkage to the DXS52 and F8C markers in Xq28. We have investigated a family with typical X-linked aqueductal stenosis, in which no linkage to these markers was present. In this family, close linkage was established to the DXS548 and FRAXA loci in Xq27.3. Our findings demonstrate that X-linked aqueductal stenosis may result from mutations at two different loci on the X chromosome. Caution is indicated in using linkage for the prenatal diagnosis of X-linked hydrocephalus.

### Introduction

Hydrocephalus unassociated with neural tube defect is a clinical entity of diverse etiology. It has a birth prevalence of  $\sim 1/1,700$  (Halliday et al. 1986). There is an excess of males affected with hydrocephalus, resulting from the existence of a well-defined X-linked recessive form of the condition (HSAS1, X-linked aqueductal stenosis; MIM 307000; McKusick 1992). This was first recognized by Bickers and Adams (1949) and reinvestigated by Edwards (1961). Since HSAS1 accounts for  $\geq 7\%$  of hydrocephalus in males (Halliday et al. 1986), it is likely to have a birth prevalence of  $\geq 1/25,000$ . Pathogenetically, HSAS1 may result from a primary CNS malformation which includes stenosis or obliteration of the aqueduct of Sylvius, connecting the third and fourth ventricles. This interpretation of the pathological features has, however, been disputed by others, who feel that aqueductal stenosis may be a secondary feature (Landrieu et al. 1979). Other principal manifestations are either hypoplasia or absence of pyramidal

tracts, enlarged ventricles, varying degrees of mental and physical handicap, and the presence of clasped thumbs.

A common clinical problem is the need to decide whether an isolated case of hydrocephalus in a male is due to HSAS1. In this situation, there is dispute over the significance to be attached to individual clinical features, including presence of clasped thumbs and radiological evidence of aqueductal stenosis, which have been claimed by some to be specific to the X-linked disorder. HSAS1 is frequently associated with a poor outcome because of severe hydrocephalus and associated CNS malformations. Perinatal death is common. However, clinical and genetic similarities with the MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs), which appears to map genetically to the same region of the X chromosome (Winter et al. 1989; Macias et al. 1992), have raised the possibility that these disorders may be allelic variants encompassing a spectrum of severity within this condition (Schrandt-Stumpel et al. 1990; Fryns et al. 1991).

For better understanding of the basis of these clinical features, detailed molecular genetic analysis of HSAS1/MASA will be required. Linkage of HSAS1 to the markers DXS52 and F8C in Xq28 was first reported by Willems et al. (1990). Subsequent reports have confirmed this linkage in additional families and refined the localization of HSAS1 to a region of  $\sim 2$  Mb between

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DXS52 and F8C (Holden et al. 1990; Orth et al. 1991; Lyonnet et al. 1992; Serville et al. 1992; Willems et al. 1992; Jouet et al. 1993). On this basis, Willems et al. (1990, 1992) have advocated the use of these Xq28 markers for prenatal diagnosis of X-linked hydrocephalus. Here, however, we report a family with "classical" X-linked aqueductal stenosis, in which the disease is not genetically linked to Xq28 markers. Linkage to the FRAXA region of Xq27.3 was demonstrated, with a maximum two-point LOD score of 2.23. These findings suggest that locus heterogeneity is a potential source of error in prenatal diagnosis of hydrocephalus by using linked markers and that, wherever possible, linkage to a particular locus should be independently established in each individual family.

## Material and Methods

### DNA Analysis

DNA was extracted from whole blood by the method of Miller et al. (1988). Digests were performed on 5–10 µg DNA, electrophoresed in 0.8% agarose gels, and blotted onto Hybond N+ in 0.4 M NaOH. Hybridizations were carried out overnight at 65°C in a mix containing 5 × SSC, 5 × Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate, 50% dextran sulfate, and 100 µg sonicated denatured herring-testes DNA/ml. Filters were washed at 65°C to a final stringency of 0.1 × SSC and autoradiographed with intensifying screens for an average of 2 d. PCR amplifications were carried out according to the recommendations of the authors listed in table 1.

### Cytogenetic Analysis

Detailed cytogenetic studies were carried out on blood from III-8. Peripheral blood lymphocytes stimulated with phytohemagglutinin were cultured in two different media (RPMI 1640 Dutch modification and Ham's F10) supplemented with 10% fetal bovine serum. Culture times of 48 and 72 h were used (the former to try to detect any abnormal cell lines unable to progress beyond the first division). Induction of rare and common fragile sites was attempted using thymidylate stress (Gosden et al. 1992). High-resolution chromosomal banding was carried out by synchronizing the cells with excess thymidine, releasing the block by three washes of prewarmed serum-free RPMI 1640, incubating for a further 3.75 h, and then adding colcemid for 15 min prior to harvesting (Gosden et al. 1992). Cytological preparations and high-resolution banding using a modified acetic saline Giemsa method were used

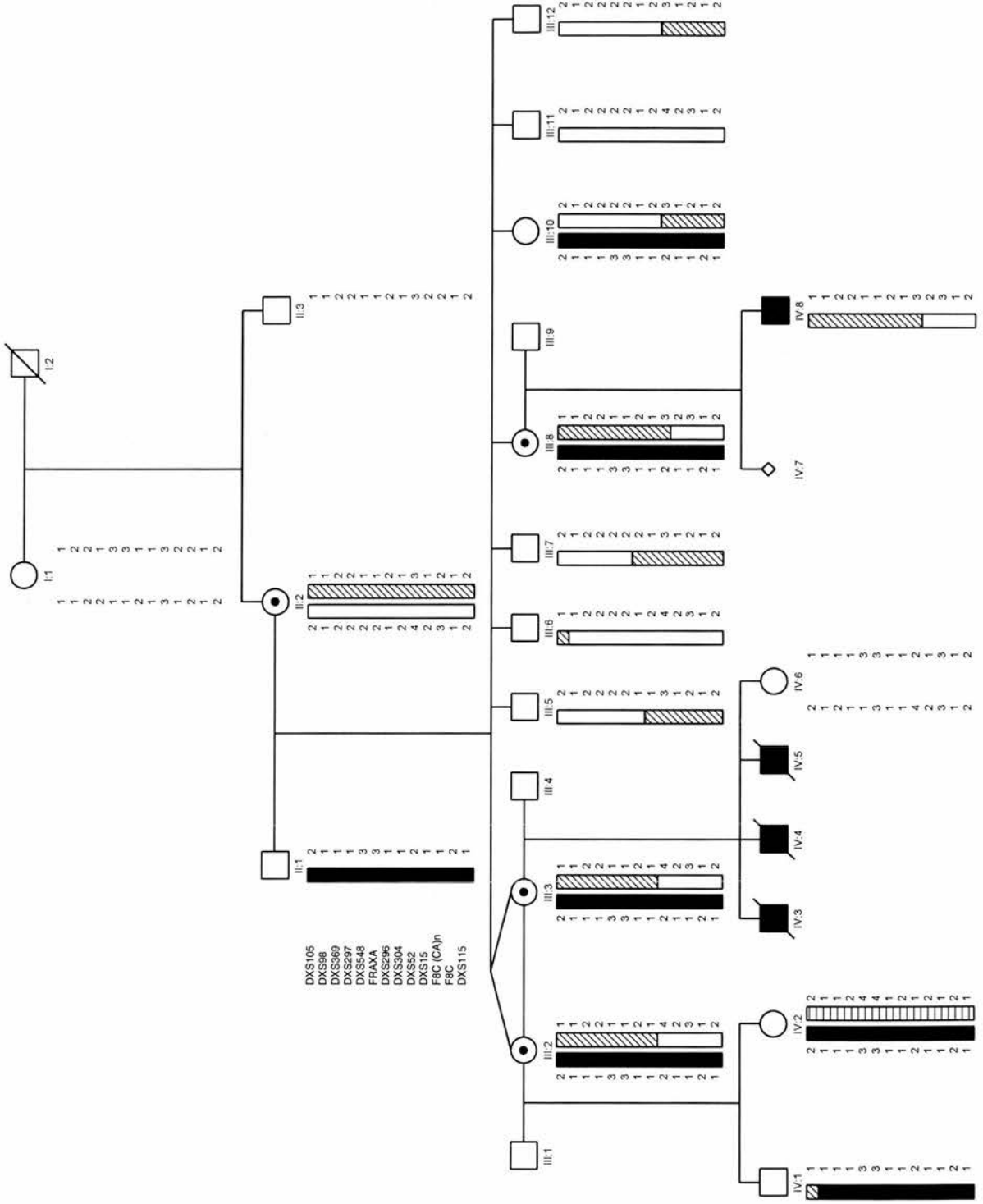
(Gosden et al. 1992). Identical chromosomal studies were also performed on 12 control females of comparable age with no history of X-linked disease. Chromosomal analysis was done by three methods: first, by using direct microscopic analysis; second, by preparing photomicrographs of X chromosomes from the proband (IV-8) and from control subjects; and finally, by using computer-aided analysis (Piper and Granum 1989), which allows alignment of very long X chromosomes from different cells, for comparison of band sizes. The computer analysis used includes special programs for "straightening" the high-resolution banded chromosomes, which allows direct comparisons of the X chromosomes from the subject and the controls, on a flexible screen. Measurements of bands of the distal part of the long arm of the X chromosome and detailed examinations for rearrangements such as inversions were undertaken. Microdensitometry of the bands of the distal portion of the long arm of the X chromosomes was carried out.

### Linkage Analyses

Two-point linkage analysis was carried out using the MLINK component of the LINKAGE program package, version 5.2 (Lathrop and Lalouel 1984). The gene frequency and new mutation rate for HSAS2 are unknown. If most X-linked hydrocephalus is due to HSAS1 (L1CAM) mutations, with a disease frequency of 1/20,000 ( $5 \times 10^{-5}$ ), HSAS2 must be rarer. We arbitrarily chose a mutation rate of  $10^{-5}$ /gamete. If male and female mutation rates are equal and the disease is reproductively lethal, this corresponds (in a population at equilibrium) to a gene frequency of  $3 \times 10^{-5}$  (Ott 1991). These figures were used for the MLINK calculation. Complete penetrance for the disease was assumed. Deceased individuals IV-3, IV-4, and IV-5 were coded as affected, as the clinical information was unambiguous. Since multilocus fingerprinting proved that III-2 and III-3 were MZ twins, they were coded as a single individual with two partners. Multipoint analyses were also carried out using the LINKMAP component of LINKAGE, analyzing two overlapping groups of six loci. VNTR alleles were condensed to a maximum of three, according to the methods of Ott (1978).

## Results

The pedigree of the family is shown in figure 1. It was verified by DNA fingerprinting (see Discussion). Four males were unambiguously affected with aqueductal stenosis, though only IV-8 could be examined in person.



**Figure 1** Pedigree of family used in this study. Affected males (◼) and obligate carriers (◉) are shown.

### Case Reports

**Case 1 (IV-3).**—The pregnancy (in 1973) was unremarkable until 32 wk gestation, when the uterus was felt to be enlarged. Gross enlargement of the fetal head was seen on abdominal X-ray at 34 wk. Spontaneous labor occurred at 36 wk; cerebrospinal fluid was drained by puncture at the time of delivery. Gross hydrocephalus was confirmed after delivery; the baby survived ~2 h. No further details were available.

**Case 2 (IV-4).**—At the end of this pregnancy (in 1974) Cesarean section was performed on account of massive hydrocephalus. The heart was beating at delivery, but the child did not breathe; no resuscitation was attempted. At autopsy it was reported that "there were abnormalities of the hands and fingers." There was massive dilatation of both cerebral ventricles, with paper-thin cortex, indicative of obstructive hydrocephalus. The brain was not fixed. However, the olfactory nerves were said to be absent, and the posterior fossa was poorly formed.

**Case 3 (IV-5).**—The third pregnancy occurred in 1980. Ultrasound scanning suggested hydrocephalus late in pregnancy, and labor was induced at 38 wk. Cesarean section was performed after failure to progress. Birth weight was 3.8 kg, occipitofrontal circumference (OFC) 42.5 cm. Apgar scores were 3 at 1 min and 3 at 5 min. The baby survived for 8 d. At autopsy, the brain was removed and fixed intact. There was gross bilateral ventricular dilatation, with only a few millimeters thickness of brain tissue overlying the convexities. The septum pellucidum and corpus callosum were absent; the latter was represented only by a thin membrane between the lateral ventricles. The anterior part of the third ventricle and the foramina of Munro were dilated. The fourth ventricle, brain stem, and cerebellum were normal, but the aqueduct was completely occluded. On histological sections, the aqueduct was represented by an anterioposterior cleft with closely apposed walls, rather than the usual triangular shape. Section through the ventricular wall showed a more or less normal cortex, a narrow sheet of white matter, and a subependymal layer of small round cells, with no visible lining epithelium.

**Case 4 (IV-8).**—The only surviving affected individual in this family was born in 1990. Ultrasound scanning at 19 wk gestation had shown no abnormality. Elective Cesarean section was performed at term. Birth weight was 4.7 kg, and Apgar scores were 6, 10, and 10 at 1, 5, and 10 min, respectively. OFC was 49 cm. Gross hydrocephalus was present at birth; cranial ultrasound

showed enormous dilatation of the lateral ventricles, mild dilatation of the third ventricle, and a normal fourth ventricle. The cerebellar vermis was normal, the cerebral cortex 8 mm thick. Thumbs were reported to be adducted at birth, but this feature was less apparent later in infancy. Opening pressure at ventricular tap was 15 mm Hg. A ventriculoperitoneal shunt was inserted at age 5 d. Cranial ultrasound at age 9 wk revealed absence of septum pellucidum and corpus callosum. Computed tomography scanning at age 8 mo confirmed these anatomical abnormalities, by detecting "a complicated midline dysraphic problem with probable partial agenesis of the corpus callosum." Sagittal and coronal craniostomosis necessitated radical craniectomy at the age of 10 mo. Formal psychological assessment at the age of 24 mo placed the boy's performance, in various areas, in the 6–10 mo range.

### Linkage Study

The clinical and pathological diagnosis of typical, severe X-linked aqueductal stenosis was therefore well established prior to the linkage study. We initially studied markers reported elsewhere (Willems et al. 1990, 1992) to be closely linked to HSAS1. For the purposes of the multipoint linkage analysis, additional markers 4D-8 (DXS98), pRN1 (DXS369), and VK23B (DXS297), in the interval DXS105–DXS296, and 767 (DXS115), distal to F8C, which were only partially informative, were included (Boggs and Nussbaum 1984; Arveiler et al. 1988; Oostra et al. 1990; Suthers et al. 1991). Markers which were uninformative in this family were VK14 (DXS292), St35-691 (DXS305), p114.12 (F8C), and F8C (*HindIII* RFLP). The DNA probes used in this study are listed in table 1. As shown in table 2, the distal Xq28 markers previously shown to be linked to HSAS1 gave negative LOD scores in our family. We therefore began to analyze more proximal long-arm markers. The order of the markers used is well established, and a consensus map of the region is shown in figure 2. The markers DXS548 and FRAXA (defined by the CGG trinucleotide repeat number) are within 150 kb of each other (Riggins et al. 1992). For both markers, the allele coded "1" in figure 1 segregates with the hydrocephalus. A peak two-point LOD score of 2.23 was calculated at a recombination fraction ( $\theta$ ) of 0. Using the genetic distances shown in figure 2, multipoint linkage calculation was performed. This indicated a most-likely location between DXS105 and DXS296, for the disease gene (fig. 2). Although we are continuing to attempt closer localization of the gene within this interval, markers analyzed so far within this region (DXS98,

**Table 1****DNA Probes Used in Present Study**

Locus (Probe)	Enzyme	Allele Size (kb)	Reference
DXS105 (55.E) .....	<i>Pst</i> I	16, 10	Mandel et al. 1986
DXS98 (4D-8) .....	<i>Msp</i> I	25, 7,8	Boggs and Nussbaum 1984
DXS369 (pRN1) .....	<i>Xmn</i> I	1.25, 1.1	Oostra et al. 1990
DXS297 (VK23B) .....	<i>Xmn</i> I	10.3, 6.6	Suthers et al. 1991
DXS548 ((CA) <sub>n</sub> ) .....	...	...	Riggins et al. 1992
FRAXA ((CGG) <sub>n</sub> ) .....	...	...	Fu et al. 1991
DXS296 (VK21A) .....	<i>Taq</i> I	10.9, 9.9	Suthers et al. 1989
DXS304 (U6.2) .....	<i>Taq</i> I	7, 3.3	Dahl et al. 1989
DXS15 (DX13) .....	<i>Bgl</i> II	5.8, 2.8	Drayna et al. 1984
DXS52 (F814) .....	<i>Bcl</i> I	VNTR	Heilig et al. 1988
F8C ((CA) <sub>n</sub> ) .....	...	...	Lalloz et al. 1991
DXS115 (767) .....	<i>Pst</i> I	1.8, 1.75	Arveiler et al. 1988

DXS369, DXS297, and DXS292) have been uninformative.

#### Cytogenetic Studies

No abnormalities were detected in the X chromosomes in the mother (III-8) of the proband, and in particular no abnormalities of the distal part of the long arm. No differences could be seen between the two X chromosomes of III-8 or between her X chromosomes and those of control subjects (which had been cultured under identical conditions) by using all the methods of analysis described.

#### Discussion

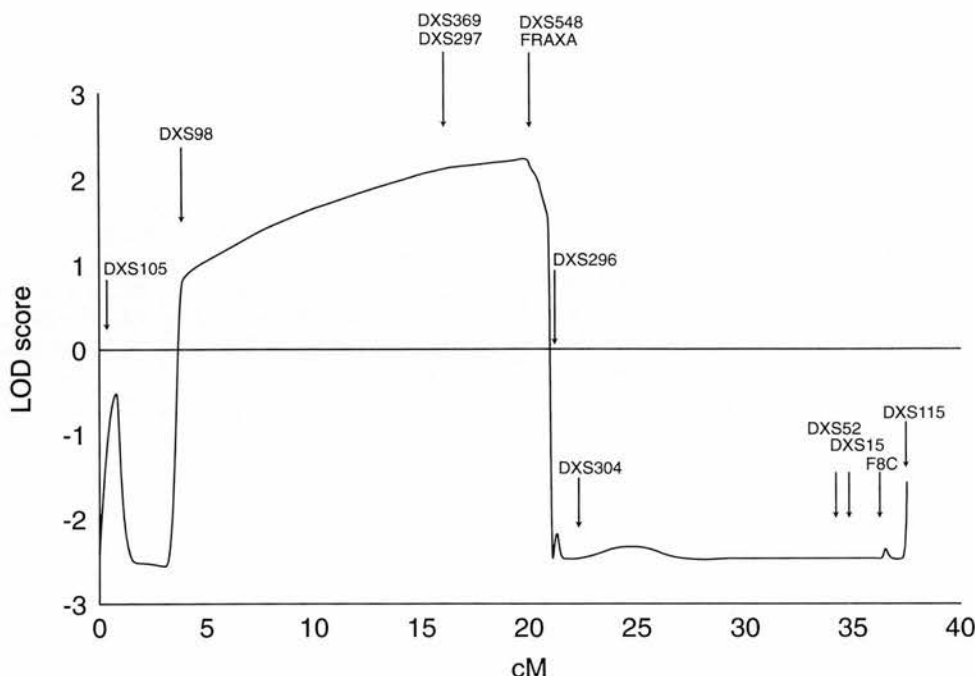
The family described here is clinically typical of X-linked hydrocephalus, with a perinatal death in three

cases and poor outcome in the surviving infant. In one case, full neuropathological study proved the existence of aqueductal stenosis; additional brain malformations were also present. The linkage heterogeneity demonstrated here cannot therefore be related to a clinically atypical picture. Negative LOD scores were obtained with the probes DXS15, DXS52, and F8C. In previous studies (Willems et al. 1990, 1992; Orth et al. 1991; Serville et al. 1992), these markers have been shown to be linked to HSAS1; recombinants between HSAS1 and DXS52 or between HSAS1 and F8C have given a likely position in the interval DXS52-F8C, for the HSAS1 gene. A candidate gene within this interval (L1CAM) has been shown to be aberrantly spliced as the result of a point mutation in one HSAS1 family (Rosenthal et al. 1992). In addition, though, to our family, it should be noted that one previously reported

**Table 2****Two-Point Linkage Analysis**

LOCUS (Probe)	LOD SCORE AT $\theta =$							$Z_{\max}$	$\theta_{\max}$
	.0	.01	.05	.1	.2	.3	.4		
DXS105 (55.E) .....	-2.47	-1.45	-.22	.21	.46	.41	.21	.46	.22
DXS548 ((CA) <sub>n</sub> ) .....	2.23	2.21	2.11	1.95	1.57	1.10	.54	2.23	.00
FRAXA ((CGG) <sub>n</sub> ) .....	2.23	2.21	2.11	1.95	1.57	1.10	.54	2.23	.00
DXS296 (VK21A) .....	-2.47	.21	.82	.98	.95	.71	.36	1.00	.13
DXS304 (U6.2) .....	-2.47	-1.94	-.77	-.30	.05	.13	.09	.13	.30
DXS52 (F814) .....	-2.47	-2.45	-1.68	-.94	-.27	.00	.07	.07	.40
DXS15 (DX13) .....	-2.47	-1.71	-.48	-.02	.28	.29	.17	.31	.25
F8C ((CA) <sub>n</sub> ) .....	-2.47	-1.72	-.52	-.09	.17	.17	.09	.18	.24





**Figure 2** Multipoint linkage analysis of X-linked hydrocephalus with Xq27-q28 markers. LINKMAP was used to calculate LOD scores from the following  $\theta$  values and map, by analyzing two overlapping groups of six markers: DXS105-.039-DXS98-.123-DXS369-.0-DXS297-.037-DXS548-.0-FRAXA-.012-DXS296-.012-DXS304-.12-DXS52-.005-DXS15-.015-F8C-.012-DXS115. The relative order of the loci is taken from Feil et al. (1990), Williamson et al. (1991), and Riggins et al. (1992). The  $\theta$  values are based on data from Wehnert et al. (1990), Richards et al. (1991), Suthers et al. (1991), and Riggins et al. (1992). The Haldane map function was used to convert  $\theta$  values to map distance.

HSAS1 family gave negative LOD scores for Xq28 markers (Orth et al. 1991; Willems et al. 1992).

The maximum possible two-point LOD score obtainable in our family, from a fully informative marker showing no recombination with the disease, is 2.71 (determined by use of a dummy marker in an MLINK calculation). In the family that we studied, the peak two-point LOD score of 2.23 at  $\theta = 0$  was obtained with the DXS548 and FRAXA (FMR-1) loci. A number of points are worth comment with regard to these data.

First, individual II-3 is the sole discordant member of the family, having the hydrocephalus-associated 1,1 haplotype for DXS548,FRAXA. He may demonstrate a recombination, occurring in I-1, between the disease and these markers, or alternatively he may indicate that II-2 represents a new mutation for X-linked hydrocephalus. Since additional markers proximal to FRAXA (DXS98, DXS369, DXS297, and DXS292) have proved uninformative in this family, we cannot at present distinguish between these alternatives; it is not possible to say whether a recombination has occurred in the interval between DXS105 (proximal to the putative HSAS2 gene) and DXS296 (distal), in the first generation.

Second, the disease segregates with an unusual CGG repeat number at the FRAXA locus. This allele (coded "1" in fig. 1) was estimated to consist of 41 CGG trinucleotides (not shown). Alleles of  $n \geq 41$  account for <2% of normal chromosomes (Fu et al. 1991). However, this allele is still within the normal size range (the range not showing any meiotic instability) (Fu et al. 1991). The faint possibility that instability of the repeat could be involved in the disease was eliminated by Southern blotting using the probe StB12.3 (*Hind*III digest) (Oberlé et al. 1991; Rousseau et al. 1991). This showed homogeneous, normal sized bands in the surviving affected boy and his mother (IV-8 and III-8).

Third, markers within Xq28 showed an unexpectedly high frequency of recombination in this family. In particular, in three cases (III-2/3, III-10, and III-12) there is recombination in the interval between DXS304 and DXS52 ( $\theta = .12$ ), and in one case (III-8) there is recombination in the interval between DXS52 and DXS15 ( $\theta = .005$ ). As expected from this observation, two-point intermarker analysis fails to show evidence for significant linkage between some pairs of loci (data not shown). Nonetheless, significantly negative LOD

scores at the true value of  $\theta$  were not observed between any adjacent pair of the markers shown in figure 2 (data not shown). Also, no obligatory double-recombination events have occurred in this family within the 38-cM interval covered by these markers. Because we initially suspected that the high number of recombinants might indicate incorrect pedigree structure, all individuals on the pedigree were rebled and reanalyzed for most of the markers. No discordant analyses were found, which eliminated sample identification errors. The entire pedigree was also analyzed by multilocus fingerprinting using the probe 33.6 (Jeffreys et al. 1985; Ferrie et al. 1991), which verified the pedigree structure as shown. (This analysis also demonstrated that III-2 and III-3 were MZ twins, as indicated.) Trivial explanations for the unusual recombination frequencies have thus been eliminated. However, we appreciate the theoretical possibility that in our family local peculiarities of the Xq27-q28 region have affected the segregation of alleles in this region. Because of this, detailed cytogenetic analysis of the carrier female III-8 was performed (see Material and Methods), with no evidence found for any abnormality of Xq28.

Case reports indicate that rapid ventricular dilatation in HSAS1 may occur only late in pregnancy or even postnatally (Jansen 1975). Since, as a result, prenatal diagnosis of X-linked hydrocephalus by obstetric ultrasound is very unsatisfactory, genetic diagnosis would be of great value. Willems et al. (1990, 1992) have advocated the use of Xq28 markers for this purpose. However, the lack of linkage to this region, in our family, suggests that caution must be exercised and that prenatal diagnosis should only be offered if linkage or mutation studies within the individual family support linkage to a particular marker. Our experience also suggests that clinical criteria are unlikely to be of any help in discriminating between genetic types of X-linked hydrocephalus.

## Acknowledgments

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## Re: Diagnostic potential of fetal renal biopsy

This interesting paper by Greco *et al.* (1993) has demonstrated that fetal renal biopsy is technically feasible, but it gave no indication that pain relief was provided for the fetuses (who were not benefited by the investigation). In our nursery, it would not be considered civilized to put a 20-gauge needle into the kidney of a 25-week infant without analgesia; why should it be acceptable for a fetus of the same age?

The question of the age at which a fetus feels pain is difficult. Thalamo-cortical connections are established by 24 weeks, and pain fibres to the brain stem and thalamus are fully myelinated by 30 weeks (Anand and Hickey, 1987; Porter, 1989). Even a second-trimester fetus moves away, at least as a reflex, from a needle tip.

I feel strongly that consideration should be given to adequate fetal analgesia in all procedures

involving invasion of the fetus itself, whether they are for diagnosis, therapy, or research.

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## Prenatal diagnosis of X-linked hydrocephalus

Serville *et al.* (1993) report the successful prediction of normal outcome in two pregnancies at 25 per cent risk for X-linked hydrocephalus (HSAS; McKusick catalogue No. 307000). They advocate the use of Xq28 polymorphisms (particularly those in the interval *DXS52-F8C*) for prenatal diagnosis of this disorder. The *HSAS1* locus is allelic with

*L1CAM*, encoding a neural cell adhesion molecule, which lies within this interval (Rosenthal *et al.*, 1992).

In one family, the prenatal diagnosis rested on firm ground, since linkage of the disease to the Xq28 markers used for prenatal diagnosis had already been established by a study of this family.



However, the second family was small, with only weak evidence in favour of Xq28 linkage. In this situation, as discussed by the authors, prenatal diagnostic accuracy depends on the supposition of locus homogeneity for *HSAS/LICAM*.

We would like to caution against unguarded application of the approach advocated by Serville *et al.* We have recently studied two Scottish families.

In one (Strain *et al.*, 1994) there have been four cases of severe X-linked hydrocephalus, with three perinatal deaths, and, in the surviving baby, severe hydrocephalus at birth with high morbidity. A full neuropathological study of one infant confirmed typical HSAS with severe aqueductal stenosis. Negative LOD scores were obtained with all Xq28 markers tested, ruling out the involvement of the Xq28 locus in this family, despite the classical clinicopathological presentation.

We have also restudied a previously reported family (Fried, 1972) in which variable mental retardation, gait abnormalities, spasticity, and, in some affected individuals, hydrocephalus are inherited in an X-linked pattern. The condition in this family bears a close resemblance to the MASA syndrome, now believed to be allelic to *HSAS/LICAM*. Again, we found no evidence for Xq28 linkage, with small negative LOD scores against all markers in this region.

In addition, one other small HSAS family reported by Willems *et al.* (1992) also exhibited recombination events between an entire Xq28 haplotype and the disease locus, but is not mentioned in the discussion by Serville *et al.*

Their own study, which Serville *et al.* cite in support of genetic homogeneity (Lyonnet *et al.*, 1992), actually only involved five families. Their impressively large family 1 (family 13 of Willems *et al.*, 1992) is linked to Xq28, with a LOD score of 4.57 against *DXS52*, but this family alone accounts for most of the total LOD score of 7.2 in the five families. Altogether, linkage data have now been reported on about 20 families (13 in Willems *et al.*, 1992; 4 in Jouet *et al.*, 1993; 3 in Lyonnet *et al.*, 1992). Only family 12 of Willems *et al.* (1992) and our own family 1 appear clearly to fit the criteria for HSAS without showing linkage to Xq28, but this alone may mean that up to 10 per cent of families could be unlinked to Xq28. This possibility was discussed by Willems *et al.* (1992). The lack of linkage to Xq28 in the Fried family (Fried, 1972; Fried and Sanger, 1973) similarly suggests caution in assessment of families where the MASA

syndrome is part of the differential diagnosis. We feel that there is an appreciable risk of error in DNA-based prenatal diagnosis of HSAS/MASA unless mutational or linkage studies have confirmed involvement of *LICAM* in the individual family.

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# Discordant segregation of Xq28 markers and a mutation in the L1 gene in a family with X linked hydrocephalus

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## Abstract

**X linked recessive hydrocephalus is the most common hereditary form of hydrocephalus. Genetic analysis indicates that the majority of cases are caused by mutations in a single gene in Xq28, recently identified as the gene for neural cell adhesion molecule L1. Genetic heterogeneity for this disorder was suggested following the description of a single large pedigree where X linked hydrocephalus showed lack of linkage to Xq28 markers flanking the L1 gene. Mutation analysis in this family shows a single base pair deletion within the coding sequence of the L1 gene that would result in truncation of the mature protein. The nature of the mutation and its segregation with the disease through the pedigree indicate that it is the cause of X linked hydrocephalus in this family. These results are at odds with data obtained through segregation of alleles for markers flanking the L1 gene. Somatic and germline mosaicism is the most plausible explanation for these data, which also provide further evidence for genetic homogeneity of X linked hydrocephalus.**

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**Key words:** X linked hydrocephalus; Xq28 markers; L1 gene.

X linked recessive hydrocephalus is characterised by enlarged cerebral ventricles, mental retardation, spastic paraplegia, and, frequently, flexion deformities of the thumbs.<sup>1</sup> Genetic linkage analysis has placed the gene for this disorder within Xq28.<sup>2</sup> Recently we and others showed that X linked hydrocephalus and the related disorders MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs) and spastic paraplegia type 1 (SPG1) result from mutations in the gene for neural cell adhesion molecule L1.<sup>3-11</sup> Linkage data indicate that X linked hydrocephalus is genetically homogeneous.<sup>12-15</sup> One exception was reported by Strain *et al*<sup>16</sup> where two recombination events were observed between Xq28 markers that flank the L1 locus and the disease gene within a single pedigree (fig 1A). Repeated sampling and DNA fingerprinting were used to eliminate the possibility of sample misidentification, and thus the existence of a second gene for X linked hydrocephalus was suggested. Germinal mosaicism, a higher than

usual recombination rate, or a chromosomal rearrangement in Xq28 could also explain these data and in view of these possibilities mutation analysis of the L1 gene was undertaken.

The 28 exons of the L1 gene from subject 19 (fig 1A) were screened using SSCP (single strand conformation polymorphism) and heteroduplex analysis<sup>6</sup> and a single mobility shift observed for exon 23. Sequencing showed deletion of a single adenosine at position 3088 within the coding sequence<sup>17</sup> (fig 2). The consequence of this mutation for L1 protein structure is illustrated in fig 3. A frameshift would lead to insertion of 72 new amino acids after amino acid 1029 of the mature protein and premature termination owing to creation of a downstream stop codon (UGA). Truncation of the protein would occur before the transmembrane domain, eliminating the potential for normal cell surface expression of L1.

This mutation was most easily visualised by heteroduplex analysis which was therefore used to show complete segregation of the deletion with the disorder within the pedigree (fig 1B). Heteroduplexes are visible for the two affected boys and four obligate carrier females, whereas none of six healthy sons of carrier mothers has the mutation. This includes the two healthy brothers (9 and 14) who had previously been shown to have inherited the same Xq28 haplotype as their carrier sisters for markers flanking the L1 gene (fig 1A). One possible explanation for the genetic results obtained with this family is gonadal mosaicism of subject 3. However, using DNA from blood samples obtained on two separate occasions, this person was repeatedly identified as a somatic carrier of the deletion.

Two lines of evidence indicate that the mutation identified in the L1 gene causes the disorder in this family. Firstly, the mutation segregates with the disease despite discordant segregation of alleles for flanking markers. Secondly, the nature of the mutation is similar to that observed in other families with severe X linked hydrocephalus. We have identified three additional mutations in three independent families that would result in truncation of the L1 protein before the transmembrane domain<sup>6</sup> (unpublished observations). All three families represent the severe end of the clinical spectrum observed for this disorder.

An explanation is required for the discordant genetic data described for this family. One possibility is the occurrence of double re-

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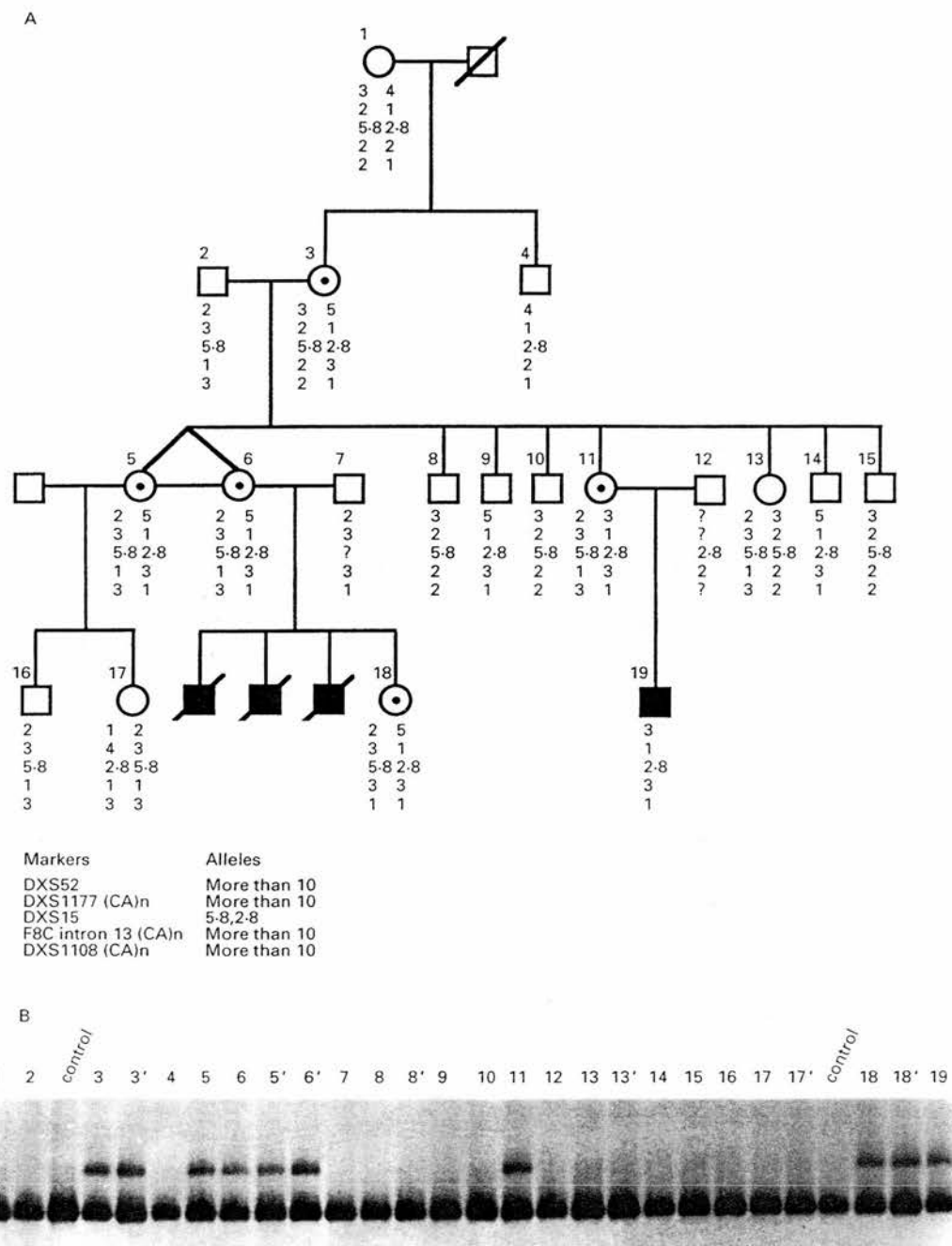


Figure 1(A) Genetic analysis showing that subjects 9 and 14 have inherited the same haplotype for markers flanking L1 as carrier females and affected boys (L1 lies between DXS15 and F8C). Data for DXS52, DXS15, and F8C are from Strain et al.<sup>10</sup> Data for DXS1177 and DXS1108 were derived for this report and confirm the original observations. (B) Segregation of the mutation throughout the pedigree as shown by heteroduplex analysis. The presence of the upper heteroduplex band is diagnostic of the presence of the 1 bp deletion.

combination events between Xq28 polymorphic markers DXS52 and F8C. The genetic distance between F8C and DXS52 is approximately 1% recombination.<sup>18</sup> The probability of two recombination events within this interval is very small and would have to have occurred twice to account for the haplotypes of subjects 9 and 14. A further unlikely explanation is that local rearrangement of L1 in this family has displaced L1 from its flanking loci which could be addressed by multicolour FISH analysis. The most parsimonious and likely explanation is that subject 3 is a somatic and germinal mosaic for the deletion owing to mutation in early embryogenesis and, as a

result, has contributed the same haplotype, with a mutation, to carrier daughters 5, 6, and 11, and without a mutation to healthy sons 9 and 14. This hypothesis is supported by the observation that there has clearly been a new mutation in the genesis of subject 3 as all haplotypes inherited by affected or carrier subjects are grandpaternal. Furthermore, combined somatic and germinal mosaicism has been well documented previously.<sup>19,20</sup>

Whatever the explanation for the disparity, these data have a bearing on genetic counselling of this family and provide further evidence for the genetic homogeneity of X linked hydrocephalus.

## Family H16

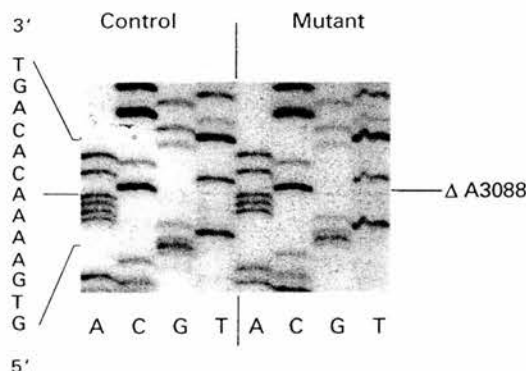


Figure 2 Sequence analysis of exon 23 from patient and healthy subject DNA. Exon 23 was amplified with primers G39 and g40 and primer G39 was used for sequencing.

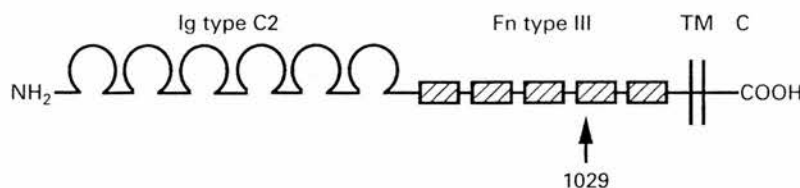


Figure 3 Structure of the L1 protein showing immunoglobulin type C2 (IgC2), fibronectin (Fn) type III, transmembrane (TM), and cytoplasmic (C) domains. The position of amino acid 1029 is indicated by an arrow.

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